STRUCTURE AND TRANSPORT FUNCTIONS OF RENAL PROXIMAL TUBULAR MEMBRANES IN ISCHEMIA INDUCED ACUTE RENAL FAILURE

SUMMARY

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

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SUMMARY

"Acute Renal Failure" is a process in which both kidneys stop their excretory function. The major causes of ARF have been identified as either ischemia or toxic insult to kidney which is manifested by oligaonuria and increased blood urea and creatinine. A variety of experimental models have been used to study ARF, but ischemia was found to be most useful one. Classical concepts regarding the cause of the loss of renal functions after ischemia include : tubular leakage across the damaged epithelium, tubular obstruction by casts formation, and decreased RBF and GFR (13). Mason (1986) proposed a new concept of vascular congestion which causes tubular obstruction as an initiator of ischemic ARF. It is reported to be produced by the aggregation of erythrocytes (13). It has been demonstrated that the tubular congestion due to vascular congestion during ischemic insult was more in the medullary region than the cortical region of the kidney (13,66,68) and was closely related with the degree of the impairment of renal functions (13,61-65). A blood reperfusion after ischemia was able to improve renal function with the removal of mostly medullary congestion (13).

In recent view the mechanism of ARF is thought to be

located at the tubular level rather than at the vascular level(6). The proximal tubule along the length of the nephron is now unequivocally considered to be the principal site of damage caused by ischemic or drug induced ARF. It is believed that obstruction material originates largely from damaged proximal tubular epithelium. During the early phase of ARF exfoliated microvillar brush border membrane (BBM) sloughed into the lumen which might be narrowed down by cell swelling (7). The casts are formed and causes tubular obstruction which lead ultimately to tubular necrosis and kidney functions are altered (1). Both proximal convoluted as well as straight tubules are greately but differentially affected (67). Mason (13) has reported that surface nephrons i.e. superficial cortical proximal convoluted tubules were less affected while deep nephrons ie S_3-subsegments of proximal straight tubules were severely damaged during ischemia. The effect of ischemia was dependent on its duration. A short-term ischemia (5-30 min) causes reversible alterations while prolonged ischemia beyond 60 min causes irreversible damage that can not be repaired even by prolonged blood reperfusion (7-9,60,112).

Renal ischemia results in profound alterations in the

morphology (203), structural integrity (7-9), metabolism and functions (60) of the proximal tubules. The brush border membrane, major functional site in the proximal tubule, is badly damaged both by ischemia and early reflow (7-9).

In ischemia-induced biohemical aberrations, inadequate supply of oxygen and metabolites resulted in severely depleted ATP levels (60,112) and altered Ca^{++}-homeostasis in the renal cortex (104-106). Both aerobic and anaerobic cellular metabolic activities were modulated (60,112,126-128) which were manifested by decreased glycolysis and TCA cycle (126-128). As a result BBM degenerates and BBM-transport functions are impaired (10-12). The activities of some BBM marker enzymes also decreases in the membranes and appeares in the urine (160-161).

In other physiologic alterations of BBM due to ischemia are loss of polarity, redistribution of Na/K ATPase, changes in lipid composition, altered membrane fluidity and loss of selective permeability for certain ions and molecules (134-139). In particular, there was a marked reduction in Na^{+}, and glucose reabsorptions (10-11). The transport of organic cations such as tetraethylammonium was also affected but only moderately (12) while alanine uptake was not altered

(11). The Na-dependent transport of Pi was not during ischemic injury except that of a report by Silverman et al. (227). However, Andreoli et al (142) recently reported that an oxidant injury by H_2O_2 in LLC-PK1, cells lowers Pi uptake in a dose dependent manner. Some of the above effects of ischemia were found to be restored by blood reflow of certain durations (7-9,60). Some limited attempts were made to ameliorate the effect of ischemic or drug induced ARF by certain hormones, drugs or metabolites (14-35,44).

In view of the above, the present studies were carried out to determine in detail the effects of ischemia and reflow on various components of the proximal tubules to gain an insight on the mechanism of ARF and its repair. The following studies were performed and the effects of ischemia and reflow were determined:

(a) On the activities of certain metabolic enzymes especially of carbohydrate metabolism in different kidney tissue zones e.g. cortex and medulla.

(b) On the activities of certain marker enzymes of proximal tubular BBM isolated from renal cortex and from superficial and juxtamedullary cortex.

(c) On the transports of 32Pi, L-(3H)-proline and D-(3H)-glucose in BBMV(s) isolated from renal cortex.

(d) On the transport of ^{32}Pi in BBMV(s) isolated from T_3-treated and/or LPD fed rats.

1. The results indicate that ischemia causes a progressive increase in serum creatinine, phospholipids, cholesterol, and inorganic Pi when produced by occlusion of left renal artery for 15-60 min. The blood reflow for different duration (15-120 min) to ischemic rats showed a reversal of ischemia-induced increase of the serum parameters and they were almost restored back towards normal values after 120 min blood reflow.

2. The activities of marker enzymes of BBM, BLM and other organelles such as lysosomes and mitrochondria were also affected by ischemia however differentially.

(a) The activities of AlkPase and GGTase in the BBMV(s) isolated from whole cortex were decreased in a duration of ischemia dependent manner and the maximum decrease was obtained after 60 min ischemia. The activity of GGTase appeared to be significantly lowered than AlkPase . However, The blood reflow upto 120 min

partially restored the activities of the enzymes (80-90% of control values) in 30 min ischemic rats. However, in 60 min ischemic rats, after 120 min reflow the recovery was much less indicating a greater damage by 60 min than 30 min ischemia (Table 4-9).

(b) Since the activities of enzymes in the homogenates were not different between control and ischemic rats, further analysis indicated that affected enzymes were actually dissociated from the membrane and become the part of the supernatent. The activities were decreased in the particle bound fraction and increased in the supernatent. The decrease in the membrane bound and increase in supernatent enzymes were in proportion to the duration of ischemia(Table 10,11).

(c) Since juxtamedullary cortex is reported to be severely damaged by ischemia than superficial cortex, BBMV(s) were isolated from these tissues from control and ischemic rats. The activities of AlkPase and GGTase were decreased linearly proportional to the time of ischemia in both BBMV-SC and BBMV-JM, however both the enzymes decreased to a greater extent in BBMV-JM than

in BBMV-SC. Reflow studies showed further decline in the activities after 15 min reflow. However prolonged reflow for 120 min resulted in the partial recovery. The reversal of GGTase activity was much slow in BBMV-JM than BBMV-SC. Again, membrane bound enzymes were decreased due to ischemia and appeared in the supernatent, while membrane bound enzymes increased with the reduction in the supernatent due to blood reflow. The appearance and disappearance of the enzymes were in proportion to duration of ischemia or reflow. Kinetic studies revealed that activities were altered largely due to Vmax effect and to a lesser extent due to Km effects(Table 12-23).

3(a) The activities of the enzymes belonging to various metabolic pathways of glucose metabolism e.g. glycolysis, TCA cycle, HMP-shunt pathway and gluconeogenesis were differentially affected by ischemia. The activities of LDH, FBPase, G6Pase and ME were rapidly increased by a brief 5 min ischemia both in the cortex and medulla, however, to different extents while the activities of MDH and G6PDH were decreased under similar conditions. An ischemia of 15 min or longer

duration e.g. 30-60 min caused decrease in the activities of all the metabolic enzymes studied. However, the decrease in the activities varied between cortex and medulla and was in proportion to the duration of ischemia(Table 28-31).

(b) The activities of above metabolic enzymes were also studied in the homogenates prepared from superficial (SC) and juxtamedu-llary cortex (JMC) after ischemia. . The pattern of the effect of ischemia was slightly different from that observed in CH and MH. The effect of different duration of ischemia resulted in the variations of certain enzymes more prominently in either of the cortical tissue. LDH was increased only in JMC after 5 min ischemia while greately decreased in SC-H compared to JMC-H after 15, 30 or 60 min ischemia. MDH was also lowered to a greater extent in SC-H than JMC-H at all ischemic time periods. Both FBPase and G6Pase similarly enhanced in SC-H and JMC-H after 5 min ischemia. The activity of FBPase decreased proportionally by 15, 30 or 60 min to similar extent in both SC-H and JMC-H. However, G6Pase activity was significantly lowered in SC-H compared to JMC-H after

prolonged ischemia (15-60 min). The activities of G6PDH and ME were increased both in SC- and JMC-H after 5 min ischemia, but the increase was greater for G6PDH. The activities of G6PDH were lowered after 15, 30° and 60 min ischemia similarly both in SC and JMC-H. However, ME activity was greately lowered in JMC than SC-H after 15, 30 and 60 min ischemia (Table 28-31).

(c) The effect of reflow on the activities of metabolic enzymes in CH and MH was determined only after 30 min ischemia. The activities of LDH and MDH were greately lowered in MH than CH by 30 min ischemia. Blood reflow causes differential effects on enzyme activities in CH and MH. After 15 min reflow the activity of LDH further declined in CH while a recovery was seen in MH and ischemia induced decrease was reduced. The medullary LDH was fully recovered after 60 min reflow but LDH in CH showed only a small recovery. The activities of MDH both in CH and MH showed the sign of recovery just after 15 min reflow but only partially recovered even after 60 min reflow. The effect of ischemia as well as reflow was greater in the cortex than medulla.

The activities of both FBPase and G6Pase (gluco-

neogenic enzymes) were lowered after 30 min ischemia although to different extents in CH and MH. However, the activities were completely recovered just after 15 min reflow and even increased far greater than control values after 60 min reflow. The activity of G-6PDH was lowered greately in CH while ME activity lowered in MH after 30 min ischemia. After 60 min reflow, the activity of G6PDH was much higher both in CH and MH than control values while the activity of ME recovered to control values after 60 min reflow (Table 34-39).

(d) The activity of BLM marker enzyme, Na/K-ATPase was also decreased but similarly both in CH and MH by ischemia and the decrease was linearly proportional to the duration of ischemia. However, the decrease in the activity was observed to be more in JMC-H than SC-H. A 60 min blood reflow to 30 min ischemic rats resulted in the partial recovery of the enzyme. The activity of acid phosphatase increased with the time of ischemia both in SC-H and JMC-H but to a greater extent in JMC-H than in SC-H (Table 32,33,40).

4. Since renal proximal tubule in general and its brush

border membrane in particular is reported to be damaged by ischemia, the transport properties of various solutes were determined especially ^{32}Pi-transport which has not been determined during ischemia and reflow.

(a) The results indicate that the transports of ^{32}Pi and L-proline were decreased differentially by renal ischemia. Na-gradient-dependent ($Na_o > Na_i$) initial uptake of ^{32}Pi declined sharply after 15 min ischemia, while L-[^{3}H]-proline uptake markedly declined both after 15 as well as 30 min ischemia. Further decrease was not obtained after 60 min ischemia and the effect of ischemia was attenuated. Na-dependent uptakes at equilibrium (120 min) were not altered after 15 or 30 min ischemia but increased after 60 min ischemia. Na-independent ($K_o > K_i$) uptakes of both ^{32}Pi and L-proline (initial and equilibrium) were higher in 30-60 min rats compared to control or 15 min ischemic rats. However, net Na-gradient dependent uptakes of ^{32}Pi (Na-dependent minus Na-independent) were declined proportionally to the duration of ischemia (15-60 min). A compensatory increase in contralateral BBMV(s) from the ischemic rats was observed both at initial uphill

phase and at the equilibrium (Table 42-45).

(b) The effect of blood reflow was studied in 30 min and 60 min ischemic rats showed that Na-dependent transports of ^{32}Pi, L-proline and/or D-glucose were affected differentially. Blood reflow for 15 min further declined the uptakes of the solutes significantly. However, 60 min reflow caused a reversal, and the transports were increased compared to 15 min reflow values. Blood reflow for 120 min resulted in a complete recovery of the transports of L-proline and D-glucose but ^{32}Pi transport was only partially restored. The blood reflow to 60 min ischemic rats, however caused further decrease in the Na-gradient dependent transports of ^{32}Pi and L-proline even after 120 min reflow. No recovery was seen in the uptake values. The results indicate that 60 min ischemia causes long term damages to transporters while 30 min ischemia causes damages which could be partially recovered after blood reflow (Table 46-50).

(c) Kinetic studies revealed that the decrease in ^{32}Pi transport by ischemia and increase due to reflow were largely dependent on Vmax alterations but to a lesser

extent on Km variations (Table 51).

(d) The transport of ^{32}Pi was further characterized during ischemia and reflow. Na-gradient dependent transport ^{32}Pi was altered only when an inward Na-gradient ($Na_o > Na_i$) was maintained. There was no effect of ischemia on ^{32}Pi-transport when Na-gradient was abolished ($Na_o = Na_i$). The Na-dependent transport of ^{32}Pi determined under different extravesicular Na-concentrations($[Na_o]$ = 20-100 mM) showed a sigmoidal relationship between the rate of ^{32}Pi uptake and $[Na_o]$ in control rats. The shape of the curves were modified during ischemia and reflow. The effect was more prominent on Vmax-values. A Hill plot transformation of the data yielded straight lines and calculated $[S]_{0.5}$ were 31.62, 35.48, 25.12, 39.81 and 31.62 for control, 30 min ischemia, after 15 min, 60 min and 120 min blood reflow, respectively. The calculated n values were 2.0, 1.67, 1.58, 1.375 and 1.33 for above respective groups. The data revealed a deviations in the interactions of Na^+ with Pi from 2:1 to less than 2 Na^+ for each Pi during ischemia and especially after 15-120 min reflow where the interaction ratio becomes almost 1:1. Thus it

appears that some intrinsic properties of 32Pi transporters in addition to other factors may have been altered during ischemia and subsequent reflow (Table 52-54).

(e) The effect of T_3 and LPD was determined on ^{32}Pi transport during ischemia and reflow. It appears from the results that both T_3-treatment and to greater extent prior feeding of LPD diet alone checked the severity of ischemic damage to ^{32}Pi transport compared to NPD-control rats. Blood reflow for 60 min resulted in enhanced recovery of ^{32}Pi transport in LPD than NPD rats. However, T_3-treatment together with feeding of LPD resulted in several fold increase in Na-dependent ^{32}Pi transport. The effect of 30 min ischemia on ^{32}Pi was very moderate and rate of ^{32}Pi uptakes were still several folds higher than control NPD rats. The blood reflow for 60 min resulted in quick and complete recovery of ^{32}Pi transport which was not achieved even after 120 min reflow in NPD rats where it was only partially restored (Table 55, 56).

In conclusion, the results of the present study evidently showed that ischemia produced by the occlusion of

renal artery markedly damages the structural integrity and may have altered metabolic and other functions of the proximal tubules. The activities of certain enzymes of carbohydrate metabolism were altered differentially in different kidney tissue zones. It appears that inadequate supply of O_2 and substrates due to termination of blood flow resulted in the alterations of metabolic activity of the cells consequently ATP levels decline in the renal tissues. As a result of ischemic injury--(lack of O_2 or ATP), the surface membrane is damaged and may have lost in the lumen. The marker enzyme components either inactivated or dissociated from the membranes and the activities declined. The membrane fluidity may have been affected as a result of changed lipid composition. Finally the transport properties of BBMV(s) alters accordingly. The effects of ischemia was duration dependent and biochemical components of the proximal tu e.g., BBM-marker enzymes (Table 4-25; Fig. 3-17), metabolic enzyme (Table 26-40; Fig 18-28) and transport of Pi, Proline and/or glucos (Table 42-56; Fig 29-44) were altered to different extent as evident from the results. The blood reflow to certain extent was able to ameliorate the effect of ischemia on different biochemical components. Prior T_3 treatment with LPD-feeding may have beneficial effects in preventing the severity of ischemic damage and in enhancing the restoration of renal transport functions during blood reflow.

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CERTIFICATE

I certify that the work embodied in this thesis is an original work done by **Mr. Syed Jalal Khundmiri** under my supervision and is suitable for the award of **Ph.D. degree** in Biochemistry.

(**Dr. A.N.K. YUSUFI**)
Reader

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SYED JALAL KHUNDMIRI

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AlkPase	Alkaline phosphatase
AMP	Adenosine-5'-monophosphate
APase	Acid phosphatase
ARF	Acute renal failure
ATP	Adenosine-5'-triphosphate
BBMV	Brush border membrane vesicles
BLM	Basolateral membrane
BSA	Bovine serum albumin
CH	Cortical homogenate
CHOL	Cholesterol
Conc.	Concentration
Cr	Creatinine
g	gram
GGTase	g-glutamyl transferase
GFR	Glomerular filtration rate
Glc	glucose
H	Homogenate
hr	hour
JMC	Juxtamedullary cortex
LAP	Leucine aminopeptidase

LPD	Low phosphorus diet
ul	Microlitre
umol	Micromole
mg	Milligram
ml	Millilitre
mM	Millimolar
min	Minute
M	Molar
N	Normal
nmol	Nanomole
no.	Number
NPD	Normal phosphorus diet
PCT	Proximal convoluted tubule
Pi	Inorganic phosphate
PL	Phospholipid
pmol	Picomole
Pro	Proline
PST	Proximal straight tubule
RBF	Renal blood flow
RF	Reflow
rmp	Revolutions per minute
RT	Room temperature
s	Second(s)

SC	Superficial cortex
SEM	Standard error of mean
SNGFR	Single nephron glomerular filtration rate
T_3	Tri-iodo thyronine
WC	Whole cortex
w/v	Weight/volume

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

GENERAL

The term "Acute Renal Failure" (ARF) denotes a dramatic clinical situation in which both the kidneys stop their excretory function within a short period of time or even immediately depending on the severity of ARF. Since the major function of the kidney is to maintain the composition of extracellular body fluids by filtration and reabsorption processes, the loss of renal function is reflected by oligoanuria and a steady rise in the concentrations of urea and creatinine in plasma (1,2). The major causes of ARF have been identified as either ischemia or toxic insult to the kidneys, resulting in an acute circulatory shock (3-12).

Acute renal failure is a process rather than a state. It begins with cellular damage initiated by primary insult and it continues until renal function and structure have essentially recovered due to reperfusion of blood or by the administration of various drugs or hormones (2,13-35). Although the time course of injury and recovery overlap and are variable due to degree of damage, nevertheless, ARF can be grossly divided into three phases, pathogenetic phase, manifestation phase and recovery phase. In the first phase, a progressive disintegration and necrosis especially of

epithelial tubular cells have been observed leading to the functional loss of the kidney which is manifested by the reduction of inulin clearance together with oligoanuria (1). In the second phase, long lasting effects are observed that severely affect the clearance of both creatinine and inulin and which can continue for several days after recovery begins, depending on the degree of renal damage. Other additional adverse effects are not observed and the recovery processes are not being initiated in this phase. In the recovery phase there is increase in urine production followed by a gradual increase in the concentrating ability of the kidney with eventual normalization of kidney function.

A variety of experimental models have been used to study ARF. In experimental animals ischemic renal damage can be produced by temporary occlusion of one or both renal arteries. Less than one hour of temporary ischemia in rats causes damage to the tubular cells and anuria but the changes, both structural and functional were reversible (1,7-9). However, prolonged occlusion of renal artery resulted in irreversible structural and functional alterations (7,60). It has been observed that unilateral

ischemic ARF causes compensatory hypertrophy of the contralateral kidney and the time taken for recovery of the kidney in the unilateral model was much slower and less complete than in the bilateral model (1). Other models of ARF are those in which the causes of the ARF were toxic agents such as heavy metals (3,4), chemicals (36-43) and drugs (5,44). Generally, ARF caused by drugs and chemicals was much more severe and irreversible and the recovery sometimes was not at all possible (1). The pathophysiologic mechanism of ARF has been investigated extensively in the last few decades (13). Although a number of structural and functional changes involved in ARF were observed, but due to the varying experimental conditions in which they were observed, and to the models, no single possible pathogenic mechanism to explain ARF could be concluded (13). Four major possible causes of ARF have been generalized which include renal vasoconstriction, glomerular permeability, tubular obstruction or tubular leakage (13). Several preventive measures have also been utilized (2,13-35); but a definite answer for the pathogenesis and its control however remain the topics for future studies.

The physiological role of the kidney in the production

of urine involves the selective reabsorption and secretion of solutes and fluids (45-48). Tubular reabsorption of sodium, the major work-function of the kidney, is dependent on active ion transport, an energy dependent process which in the kidney is primarily dependent on supplied ATP by oxidative metabolism (49-53). In fact, most of the metabolic work of the renal cell is directed towards the production of ATP for the support of active reabsorption of ions and solutes (54,55). A close relationship has been observed between ion transport and cell metabolism in extension with the O_2 consumption in the kidney (54,55). The oxygen tension or consumption has been demonstrated to be different in the nephronal subsegments distributed in metabolically different kidney tissues such as the cortex and medulla (50,53,56-59). ARF due to ischemia and toxic insult, resulted in the depressed metabolic activity because of limited O_2 supply through the blood, curtailing energy production and leading to the loss of active reabsorption and secretion processes in the kidney (10-12, 37-39,60). The present studies are carefully designed to gain an insight into the mechanism by which the experimental ischemia causes damage to the structure and the functions of the kidney in general, and the proximal tubule, a major site for kidney function, in

particular. The activities of certain metabolic enzymes, the enzymes of the luminal membrane of the proximal tubules and the transports of various ions, minerals and solutes were determined after ischemia and reperfusion of blood to enable to understand the mechanism of ARF.

Pathophysiology of Acute Renal Failure

Ischemia or toxic agents are known to cause acute and/or chronic renal failure leading to the partial or total loss of normal excretory functions of the kidney. The acute renal failure is accompanied by a simultaneous but steady rise in plasma creatinine and urea concentration (1,2). Classical concepts regarding the cause of the loss of renal functions after ischemia included tubular leakage across the damaged epithelium, tubular obstruction by cast formation or interstitial compression, a decrease in renal blood flow (RBF), and glomerular filtration rate (GFR) or glomerular membrane permeability (13). However, due to lack of support for a satisfactory explanation for the loss of renal function following ischemia, some of the above factors were not considered as the only basis for initiating the damage caused by ischemia. Raised intratubular pressure indicative of tubular obstruction was not consistently present (61). A

decrease in RBF was frequently observed but was often lacking (62) and a reduction in glomerular permeability was able to explain only a depression in filtration rate and not a decrease in blood flow or tubular function (63-65). Thus, the extensive research of the past 40 years using morphologic, clearance and micropuncture techniques were unable to provide a universal view regarding the mechanism for the pathogenesis of ARF. The other reason for not reaching a consensus could be the explanations provided were based mostly on structural alterations rather than on functional ones. Mason in 1986 (13) proposed a new concept for the problem. His view that vascular congestion occurred due to ischemia was an appropriate answer to the loss of renal functions. Mason et al (66,67) and others demonstrated that the tubular congestion to a greater degree in the medulla than in the cortex due to ischemia, was due to the aggregation of erythrocytes (5,66,68). These workers were also able to record a closely related variation with that of the ischemic impairment of renal functions (13,61-65). It was also observed that reduction in the medullary congestion due to blood reperfusion was able to improve renal functions with concomitant improvement in RBF (67,69). This could be possible primarily due to the increasing filtration rate and

delivery of filtered fluid to the tubule and secondly by improving the delivery of oxygen and substrates to the tubular epithelium which were reduced due to ischemia (13).

The above concept of vascular congestion involved in the ischemic damage was very well supported by various morphologic as well as functional features observed during ARF (13). This could be also useful to explain certain basis for the prevention or the treatment of ARF. The morphologic explanation for vascular congestion was based on the following features. The congestion was caused primarily by the reversible aggregation of erythrocytes (66). It has occurred due to the uptake of fluids into ischemic cells from the plasma leading to cell swelling consistently observed during ischemia. It has been observed that congestion occurred similarly both in the medullary and cortical regions, however, to a greater extent in the medullary region due to sluggish blood flow (13). It has been also demonstrated by reflow studies that congestion produced due to ischemic or toxic insult disappears from the cortical region but remained for a longer duration in the medullary region (70). Patchy cortical necrosis (71) often segmental in nature (71) was found to be another

morphological feature observed both in the experimental model (71) and in the shock kidney (72). Finally, tubular obstruction was also considered to be the part of vascular congestion (73-75) that has occurred mainly due to the generation of tubular cast. Thus it appears from the above observations that vascular congestion may be the only one of the factors responsible for cell necrosis, cast formation and tubular obstruction that may lead to ARF.

Tubular Obstruction

In the recent view the mechanism of ARF is thought to be located more at the tubular level than at the vascular level. However, the very same phenomenon was first proposed by Ponfick in 1875 (76). He found that nephrons and collecting ducts were filled with precipitates of chromoproteins and suggested that casts may cause tubular obstruction and in this way may be an important factor in the pathophysiology of ARF. Indeed cast formation and/or tubular obstruction appears to play a definitive role in the reduction of GFR as has been observed in most experimental models particularly in ischemic ARF (77) and has also been demonstrated in mercuric chloride (78), gentamicin (79), and myohemoglobinuric (80) ARF. Dibona (81) later noted a strong

correlation between cast formation and blood urea nitrogen (BUN) concentrations. Cushner et al (82) observed a strong inverse correlation between the number of casts and the inulin clearance. Richards and Dibona (83) also found a significant correlation between the number of casts and serum creatinine in glycerol model of ARF and Solez et al (84) noted the same in pedicle clamp model of ARF.

The evidence for a functional role of casts and/or tubular obstruction in the pathophysiology of ARF comes mainly from morphologic and micropuncture studies. The proximal tubule along the length of the nephron is now unequivocally considered to be the principal site of damage in experimental or drug induced ARF, that is characterized by the progression of the well defined events detailed as follows :

It is believed that obstructive material originates largely from damaged proximal tubular epithelium. During the early phase of ARF, exfoliated brush border microvilli sloughed into the lumen which might be narrowed down by cell swelling. During the manifestation phase, casts of compact cell debris and hyalin cylinders and other damaged materials generate the obstruction not only in the proximal tubular

lumen, but also in more distal nephron segments affecting the reabsorptive and concentrating abilities of the ischemic kidneys due to ultimate cell necrosis (1). Donohoe et al (85) in their studies on ischemic ARF observed that occlusion of renal artery for short durations (15 min) had little morphologic or functional alterations. However, 25 min ischemia with early reflow period showed cellular necrosis and obstruction which was dependent on the duration of ischemia as well as on the duration of reperfusion (77). Both proximal convoluted as well as straight tubules were greatly, but differentially affected (77). This work confirmed the earlier findings of Tanner and Sophasan (86) in which they also noted proximal tubular dilation and obstruction in the early reflow period and the presence of distal cast formation at 24 hr. In volume expanded rats, Arhendshorst (87) also reported similar findings and obstruction was observed but without variation in the GFR after 24 hr of reflow, which was also confirmed by Tanner and Steinhausen (88) in later studies. Additional support for intratubular obstruction playing an important role in the pathophysiology of ischemic ARF comes from comparison of measurements of single nephron glomerular filtration rate (SNGFR) and total kidney GFR. Tanner et al (89) noted that

while SNGFR declined only by 30%, the whole kidney GFR was reduced by 90%. This discrepancy between the SNGFR and GFR might have been due to the location of the nephron from which SNGFR was determined. Mason et al (90) reported that SNGFR measured in the superficial nephron following renal ischemia may not be representative of deeper nephrons because they were partly protected by ambient oxygen concentration during the ischemia and that deeper nephrons not exposed to oxygen were more severely affected. Significant amounts of back leak may also be another reason for the above discrepancies.

In summary, tubular obstruction most likely plays a major role in the pathophysiology of ARF. Whatever may be the exact mechanism for the pathophysiologic changes observed in ARF, proximal tubular cell injury might be the major cause for the loss of renal function after ischemic or toxic ARF (90). Trump et al (91,92) have described detailed morphologic changes which occurred due to injury to renal proximal tubules in ischemic ARF.

Cellular and Molecular (Functional) Mechanism of Nephrotoxic or Ischemic ARF

a) Cellular events : It is now becoming increasingly

clear that the final pathway for the development of both ischemic and nephrotoxic ARF is renal tubular cell injury (93,94). It occurs mostly due to segmental necrosis that might initiate a variety of factors responsible at the nephron level and in several stages for excretory failure of the kidney. A better understanding of the cellular and molecular events that occur after the cell is subjected to injurious stimulus may remain incomplete unless related preventive or controlling measures are also devised for the explanation of the damage (71,95). An understanding of the mechanism by which the cells repair the injury and regenerate, would permit a more rational approach towards treatment or prevention.

Trump et al (91) based on his studies has proposed **seven stages** of cellular injury which occur during ischemia. Some of them were reversible and a few were irreversible depending upon the duration and type of insult and susceptibility of the particular cell to the injurious stimulus. Following an ischemic injury, in **stage one**, cellular oxygen tension decreases with a rapid decline in mitochondrial phosphorylation and cellular ATP levels (96,97) inducing anaerobic glycolysis which in turn causes a

decrease in cytosolic pH and subsequent clumping of nuclear chromatin. The decreased ATP levels also leads to impairment of cell ion pumping systems initiating the redistribution of ions such as Na^{+}, K^{+}, and Ca^{++}.

In **stage 2**, the redistribution of ions and water leads to swelling of the endoplasmic reticulum (ER) lumen and accumulation of ions within mitochondria (98,99), causing changes in cell shape due to malfunctioning of the microtubules and microfilaments because of the presence of higher Ca^{++} ions.

Stage 3 was characterized by intracristal swelling of mitochondria and dilation of ER due to continued ion shifts. The imbalance of ions thus created was associated with loss of both free and bound polysomes causing cessation of protein synthesis. Thus far the consequential changes due to ischemia were considered to be reversible.

Stage 4, was considered as a transition phase that initiates cell death. Some mitochondria at this stage begin to show swelling of their inner matrical compartments with small dense aggregates, perhaps of, reversible denatured matrix proteins. The continued increase in the cytosolic

Ca++ ion result in phospholipase activation, causing breakdown of phospholipids and the accumulation of free fatty acids.

The later remaining **stages** were considered to be irreversible. In **stage 5**, high amplitude swelling of all mitochondria was observed and large flocculent densities either due to proteinaceous material (100) or lipids (101) appeared within the mitochondrial matrix. There was an increase in the membrane permeability with diffusion of enzymes, lipids and other cell factors. Large membrane discontinuation was seen and chromatin digestion also occurred at this stage. Necrosis with digestion of cellular constituents characterized **stage 6**. Karyolysis, lysosomal disruption and changes in staining properties of cellular components were also apparent at this stage. The final degradation of the cell and the appearance of myelin figures and the total absence of enzymatic activity signaled the final stage i.e. **stage 7**.

The progression of cell death appears to involve atleast two mechanisms (102,103). The first mechanism which was independent of extracellular Ca^{++} ions, involves disruption in the integrity of the plasma membrane either by

ischemia or by direct cell membrane damage. The second mechanism was the functional consequence of an injurious stimulus, and most likely involved the influx of extracellular calcium down its steep concentration gradient. It appears from these studies that the increased cytosolic Ca^{++} may in some manner initiate many of the catabolic processes that effect the transition from a reversible type injury to an irreversible one that inevitably leads to necrosis. Based on the above observations and others (104) Ca^{++} ions were implicated as being one of the major factors of ischemic and toxic ARF (105,106) but a definitive role was not clearly identified.

Thus, it appears that ischemia directly leads to decline in the rate of oxidative phosphorylation due to lack of availability of oxygen. A fall in cellular ATP level develops. Ischemia was also shown to promote redistribution of intra-cellular pools of ions especially that of the Ca^{++} ion pools and resulted in phospholipase activation and phospholipid degeneration leading to a net loss of membrane phospholipids and hence the membrane integrity and functional capability.

b) Biochemical (Molecular) events : Recently, Humes and

Liu (6) suggested that the physiologic changes which occur during ischemic or nephrotoxic insult are due to biochemical alterations at the molecular level. The sequence of events between the initial biochemical aberrations and the observed renal functional impairment were undoubtedly complicated and data on the biochemical changes of any model of ARF are lacking. It is clearly evident that an inadequate supply of oxygen and metabolic substrate to renal tissue is the initial major biochemical aberration in ischemic ARF. The proximal tubule is largely dependent on oxidative metabolism for generation of the energy (or ATP). However, distal portions of the nephrons are able to synthesize ATP under the conditions of limited oxygen availability (56,57,107).

It has been known for long that pO_2 is much higher in the outer cortical nephronal subsegment such as the proximal convoluted tubule compared to juxtamedullary nephronal segments such as proximal straight tubule (pars recta), MAL, or MCT (108-111).

Carbohydrates (glycogen or glucose) appear to be the major energy reserves in the kidney besides nucleotide triphosphates. Glycogen has been reported to be negligible in the proximal tubule, whereas substantial glucose is

reported (112). In contrast distal structures contain major concentrations of both glycogen and glucose (112). Glucose has been shown to be oxidized as well as produced by the kidney although differentially in different tissue zones and nephronal segments in the kidney. The oxidation of glucose in the kidney has been shown to occur by different metabolic pathways depending on the location and types of particular nephron segments in the kidney. The enzymes belonging to the different metabolic pathways are found to be present and distributed differentially in the kidney. The renal medulla and the nephron segments located therein is the major region for the production of lactate from glucose by glycolysis (57,113-115), while the oxidative conversion of glucose to CO_2 (by TCA cycle) is shown in the renal cortex and the nephron segments located therein eg. PCT (57,115). Glucose is also partially oxidized via HMP shunt pathway (56,57,115-121). On the other hand glucose is known to be produced in the kidney by gluconeogenesis especially in the proximal convoluted tubules (57,107,122-125). It was further demonstrated that although the net uptake of glucose was higher in renal medulla than the cortex, the proportion of glucose oxidized to CO_2 was higher in the cortex than medulla, so that over all glucose oxidation appeared to be

much greater in the renal cortex than in the medulla (57,113-115). Definite information about glucose oxidation rates in defined nephron subsegments is limited at present (115). However, the distribution of numerous enzymes belonging to various metabolic pathways has been demonstrated in different subsegments of the nephrons (57). Available evidences have clearly indicated the predominance of the glycolysis in renal medulla while oxidative metabolism was more prevalent in the renal cortex (57,115). Similarly the occurrence of HMP shunt pathway and gluconeogenesis has been differentially demonstrated in the cortex and medulla or in different nephron segments (57,115). Thus the quantum of energy produced by the above pathways in different kidney tissue zones or in different nephron segment would directly or indirectly determine the functioning of that particular part of the kidney.

At present the information regarding the metabolic activity via various metabolic pathways or the activities of various enzymes linking to different metabolic pathways in ischemic or nephrotoxic ARF is not available in detail except that of few scattered reports (57,107-115). In the first report Vogt et al (1968) have demonstrated that

ischemia caused reversible changes upto 25 min period and beyond this time period (>60 min) the changes were mostly irreversible. The authors observed that renal cortical ATP falls rapidly but could be restored within 10-20 min by blood reflow in short duration ischemia and not in prolonged ischemic ARF. Lactic acid levels were found to be increased in proportion to the time of ischemia (60). Randall Jr. (1969) however demonstrated that ischemia due to renal veins was more damaging than due to renal artery ischemia and that the metabolism was greatly affected in the medullary homogenate than cortical homogenate (126). The activities of MDH and ICDH, the TCA cycle enzymes were also found to be reduced by ischemia (126). Hems and Brosnan (1970) studied the effect of ischemia on the content of metabolites in rat liver and kidney (127). A brief ischemia of upto 5 min caused the accumulation of lactate and fructose-6-diphosphate (FDP) with simultaneous decline of glucose and glucose-6-P. The content of a-Ketoglutarate (a-KG), pyruvate, oxaloacetate (OAA) and glutamine together with ATP was decreased while that of glutamate was increased during ischemia (127). In a more recent study Bastin et al (1987) have determined the effect of brief ischemia (upto 2 min) on the energy reserves of different nephron segments

(112). The effect was same as reported in the previous studies by Hems and Brosnan (127) in the whole kidney or isolated tubules. The ATP declined most rapidly in the proximal convoluted (PCT), proximal straight tubules (PST) and distal convoluted tubule (DCT) and most slowly in the papillary segments. Glucose consumption and lactate production were most rapid in distal portions and glycogen hydrolysis was most slow in the proximal tubules (PCT and PST) (112). Similar results of increased lactate production by virtue of increased LDH activity were also obtained by Dickman and Mandel (1991) during short-term anoxia and hypoxia in the proximal tubules (128). Thus far it has been demonstrated that brief ischemia affect the content of various metabolites and ATP, however, the enzymes of various pathways and actual metabolic rates were not studied especially with respect to short-term and long-term ischemic ARF.

c) Functional Events at membrane level : The plasma membrane is the cell boundary which interacts with other cells and the external milieu and is also the site for transport of various ions and solutes (46,47,129). The structural and functional integrity of plasma membrane

appeared to be essential for continued cellular homeostatis and viability. An alteration in the transport functions of the plasma membrane can be considered as a prime initiator of ARF, although it seems to be secondary to a decrease in the metabolic energy atleast in the case of ischemic ARF. Nephrotoxins are also known to interact with the plasma membrane altering it in such a way that cell death ensues. Further structural alterations of the plasma membrane may also involve in the manifestation of ARF. The effect of ischemia has been manifested in rats by the reversible disappearance of brush border microvilli after 30 min of ischemia (130,131) and irreversible interiorization of microvilli and loss into the lumen (7,132) leading to cellular necrosis and ARF when ischemic period lasted for more than 60 min. (8). Loss in the membrane polarity of both apical and basolateral membrane domains, alteration in apical terminal web actin micro-filament (133), changes in the activities of specific marker enzymes (8,9) and reduction in the vectorial transport functions (10-12) were observed due to ischemic ARF. The alterations in vectorial transport functions were reported to be related to the redistribution of surface membrane phospholipids (11,134) and domain-specific apical and basolateral membrane enzymes

(10,135,136). According to Molitoris and coworkers (10-12,137) ischemia-induced redistribution of Na^+-K^+ ATPase to the apical membrane in renal proximal tubule cells was associated with reduced Na^+ reabsorption, apparently due to Na^+/K^+ ATPase pumping back into the lumen (136) which has been accounted for the loss in the membrane polarity. It has been also suggested that ischemia in renal proximal tubule cells resulted in the rapid duration dependent opening of the tight junctions concurrently with the redistribution of surface membrane phospholipids and Na^+/K^+ ATPase to the alternate surface membrane domain (138). Microfilament disruption was also suggested to be involved in ischemic cell injury, as disruption of terminal web and microvillar actin microfilaments occurs early and in a time dependent fashion (139). Disruption of microvillar core microfilaments might be responsible for loss of apical membrane and the markedly altered morphology of apical microvilli seen after ischemic injury (137). Microfilament disruption during ischemia might be the result of cellular ATP depletion as metabolic inhibitors resulted in breakdown of actin microfilaments in cell culture studies (140). These data thus indicate a relationship between the cell viability to maintain surface membrane polarity and the disruption of

lateral cell-cell attachments and the actin cytoskeleton, since under these aberrant conditions, apical and basolateral proteins and lipids may be capable of lateral diffusion within the plane of the membrane bilayer and might have diffused into the alternate domain, resulting in loss of surface membrane polarity and finally functional capabilities. Renal tubular epithelial cells also respond to oxidant injury with an early fall in ATP levels, while cell detachment and lytic injury occur as later events (141).

In a recent study Andreoli et al (142) reported the decline in Na^+ dependent glucose transport in LLC-PK1 cells in response to oxidant injury, along with decrease in ATP levels and Na^+-K^+ ATPase activity. Transport function within a membrane depends on structural integrity of the transporter and a normal membrane lipid environment to support transporter activity (133). Therefore, oxidant injury appears to be one of the causes of ARF due to ischemia (143,144) because proteins and lipids are susceptible to oxidant injury (29,145-147), and thus desrupting the transporter function at the membrane level. Additionally, oxidation of critical amino acids may also occur within the transporter or alterations of the lipid

environment, resulting from lipid peroxidation.

d) Protection and prevention of ARF : It appears from all of the above discussion that the pathophysiology and/or biochemistry of ARF is not yet fully clear. A partial repair, prevention and/or protection would also be a more rational approach for better understanding the pathogenesis as well as for the treatment of ARF. Reperfusion of blood, and certain chemicals, drugs or hormones have been used in recent past demonstrating a partial recovery from ischemic and toxic shock (2,14,15-35). Some attempts have been made in the recent past to reverse the drug or ischemia-induced alterations by using glucose, essential and non-essential amino acids and their a-keto derivatives which could increase the survival rate after the ARF episode (150). Solez et al (151) reported a beneficial effect of propranolol and clonidine. Also mannitol, furosemide, dopamine, prostaglandins, and bradykinin have been shown to prevent the functional defect in some models of ARF. Recently, calcium channel blockers like verapamil have been included in the above group (152-154).

Since ischemia consequently causes depletion of tissue levels of ATP, ADP and AMP (15), it has been proposed that

restoration of ATP synthesis by the ischemic kidney definitely can have a beneficial effect. Seigel et al (16,17) showed that administration of adenine nucleotides (ATP, ADP and AMP) combined with magnesium chloride ameliorates the renal function after the initiation of acute renal failure. Various other agents like epidermal growth factor, EGF (18-20,33), endothelin (2,21), atrial natriuretic factor (22,23), defibrotide (25,26), glycine and glutathione (42). Thyroxine-T_4 (4,24) and insulin like growth factor IGF1 (14,32) have been shown to control renal failure either due to toxic insult and/or ischemia. Various hydroxyl radical scavengers like superoxide dismutase (28), dimethylthiourea (DMTU) and allopurinol (29-31,34) when administered just prior to the ischemic event have been also reported to ameliorate renal function.

Above attempts which were made to ameliorate the function of ARF kidney,generally resulted in the improvement of GFR and RBF, decreases serum Cr, and BUN with increased inulin and creatinine clearance and in the enhancement of cellular regeneration (2,14,15-35,155). The above efforts, however, failed to provide any generalized mechanism for the damage or repair of the ARF kidney in particular.

SCOPE OF THE THESIS

Renal ischemia results in profound alterations in tubular cell functions (89,94), metabolism (60,222) and structural integrity (130,132). Studies involving ischemic and/or toxic ARF showed that renal proximal tubular cells are especially affected (7) and that these tubular cells undergo dynamic transformations especially during early reflow leading to renal dysfunction (7,156). The damage that occurred to proximal tubular cells, the main functional site where most of the fluid, ions and molecules are reabsorbed (129,157), are essentially not known unless a reflow is followed by the ischemic insult. Both the early damage due to ischemia or early recovery due to reflow are difficult to follow. However, their knowledge is very much essential for better understanding of the pathophysiology of ARF. It has been suggested that the proximal tubular brush border membrane (BBM) is badly affected by ischemic or toxic insult and is mainly responsible for the loss of excretory kidney functions because of varied reasons (7). The effect of ischemia on the proximal tubular cells (85,158) has been manifested in rats mainly by the disappearance of brush border microvilli (130,131), or by irreversible

interiorization of microvilli and loss into the lumen (132) leading to cellular necrosis and ARF (8). The proximal tubular cell membrane has been found to be histologically regenerated after ischemia upon reperfusion of blood within 6 hr. Physiologic alterations of BBM due to ischemia implicating surface membrane dysfunction include, loss of polarity, changes in the fluidity (9-11,133,136-139), loss of selective permeability (85), reductions in the activity of BBM associated enzymes (8) and decrease in proximal tubular fluid, (159), sodium (10), glucose (11) and cation (12), reabsorption. Paddock et al (8) using a 25-30 min clamp model have demonstrated a loss of 50% activities of BBM-enzymes: alkaline phosphatase and g-glutamyl transferase, decreased incorporation of ^{3}H-leucine into BBM proteins (also protein content) and increased incorporation of ^{14}C-choline into BBM-phospholipids (8). The reported loss in BBM-biochemical parameters (structural component) has been shown to be partially recovered after reflow of blood to ischemic rats (8). It was further noted that ischemia causes a decline in the BBM glycoprotein content which could be recovered after reflow as measured by ^{3}H-fucose incorporation into membrane glycoproteins indicating that glycoprotein recycling occurs to a large extent in the

ischemic kidney (9). In another study, Herminghusen et al (160) and Desmouliere and Cambar (161) observed an increase in urinary g-glutamyl transferase activity in ischemic rats indicating the alterations in the brush border membrane and the sloughing of the enzyme components to accumulate in the urine. Increased fractional sodium excretion together with increased renal oxygen consumption was also observed (160). Thus, the above studies clearly indicate that ischemia together with early reflow causes alterations in the structure and metabolic or transport functions of the proximal tubule cell including its membrane.

Heterogeneity of the proximal tubule in terms of both structure and function is well known (162,163). It has been shown that proximal tubules which consists of S_1 (pars convoluta), S_2 and S_3 (pars recta) subsegments, differ in the distribution of marker enzymes (162,164), reabsorptive properties of various solutes (165) and differential response to various drugs (166), hormones (167-169) and dietary stresses (170-172). Mason (13) has reported that these nephron subsegments were also differentially affected by ischemia. While the surface nephrons (S_1-subsegment) showed relatively less effect, the deeper nephrons (S_3-

subsegment) were severely damaged (13). It has been further reported that nephron functions, early after ischemia, were suppressed to a small extent in surface nephron, were more affected in the middle nephrons and particularly severely depressed in the deep ones (13). Gentamicin and other aminoglycoside antibiotics induced toxic insult, showed specific effects in proximal convoluted tubular (PCT) segments (166,173-175) while proximal straight tubule (PST-*pars recta*) segment was shown to be more sensitive to oxygen deprivation, ischemic or toxic insult due to urynylnitrate. Recently, Ruegg and Mandel (176) reported that PCT subsegment was more responsive to anoxia-induced injury under *in vitro* conditions contrasting to the *in vivo* conditions in which PST was considered to be maximally damaged due to ischemia. However, topographic studies performed after ischemia with reflow in rat kidney showed a different ideology about the pathogenesis of ARF in proximal tubules. Shanley *et al* (177) described that the effect of ischemia-reflow combine on proximal tubular segments was dependent both on the duration of ischemia as well as on the duration of reflow. S_1 subsegments were selectively sensitive to short-term ischemia, while S_3 subsegments were particularly sensitive to long term ischemic effects. These

authors suggested the possibility of two distinct mechanisms of proximal tubule necrosis in ischemia and reflow. One is based on an intrinsic vulnerability to ischemia per se, due to a limited capacity for anaerobic glycolysis and dependence on oxidative metabolism (164). Studies with metabolic inhibitors suggest that this dependence is most marked in S_1 and least marked in S_3-subsegments (178). The second mechanism relates to inadequacy of oxygenation during the reflow period and resulted in a distribution of injury indicative of a relationship to perivascular gradients of oxygenation. The mechanism is probably the major determinant of the vulnerability of the S_3-subsegment in the ischemia-reflow model (177). The oxygenation dependent changes may include the curtailment of various metabolic activity and the depletion of ATP required for several functional activities including transport functions (10-12,60,125-128). Several other factors included in causing damage to proximal tubules besides oxygen availability were calcium ions (151-153) and oxygen and hydroxyl free radicals formation (144). However, Molitoris et al (9-11,133,134,136-139) has implicated variations in lipid contents or fluidity of the membrane as one of the major aspirant for ischemic and toxic ARF.

ischemic state.

In transport functions, reduction in the Na-dependent glucose (10,11) and organic cations (12) has been observed in ischemia induced ARF. The transport of Pi, which involves in the maintenance of energy for many renal functions (46,47,129) has not been studied in detail under reversible and irreversible ischemic conditions. The transport of Pi in renal proximal tubule is known to be regulated at the brush border membrane site (129). The feeding of low Pi diet (LPD) and the treatment of thyroid hormones resulted in the increased transport of Pi by differential mechanisms (179). Thyroxine has been demonstrated to be beneficial in drug-induced ARF (4,24).

In view of the above discussion, the present work was carefully designed and undertaken to study the damage caused by ischemia to the structure and functions of the renal cortical proximal tubules. For better understanding of the mechanism of the pathogenesis of ischemia-induced ARF and its possible reversal, the following studies were performed.

1) The activities of various enzymes of metabolic importance especially related to energy metabolism (in particular of glucose metabolism) were determined in different tissue zones of the kidney e.g. cortex and medulla isolated from ischemic and reperfused rats

under varying conditions.

2) The activities of some marker enzymes considered to be of structural and functional importance of the proximal tubular BBM e.g. AlkPase and GGTase, and others belonging to some other organelles were also determined during ischemia and blood reflow in kidney tissue zones, in SC- and JMC- tissues and also in the BBMV(s) isolated from above rats.

3) Transports of Pi (in particular) together with the transports of amino acids (proline) and sugars (glucose) were determined and further characterized in the BBMV(s) isolated from ischemic and blood reperfused rats.

4) The protective/preventive effect of thyroid hormone (T_3) and LPD were explored on BBM Pi transport affected by ischemia.

The central hypothesis of the present work, therefore is to study the effect of ischemia and subsequent reflow on (1) The activities

The present studies showed specific alterations due to ischemia and blood reflow on the above parameters. These studies would be helpful in furthering the understanding of the pathogenesis of ischemia-induced ARF and its possible prevention or protection.

(X) of BBM-marker enzymes (2) The activities of certain enzymes of carbohydrate metabolism and (3) The transport capabilities especially that of Pi in BBM-isolated from renal cortical proximal tubules to gain a broader and insightful knowledge about the mechanism of pathogenecity of ARF.

MATERIAL & METHODS

MATERIAL

Animals :

Adult Albino rats (Wistar strain) were purchased from Experimental Animal Facility Centre, AIIMS, New Delhi and Jamia Hamdard University, New Delhi, India.

Substrates of carbohydrate metabolism enzymes :

D-Glucose-6-phosphate for glucose-6-phosphate dehydrogenase and glucose-6-phosphatase. Pyruvate-Na for lactate dehydrogenase, oxaloacetate for malate dehydrogenase, L-malic acid for malic enzyme, fructose 1,6 diphosphate for fructose diphosphatase, p-nitrophenyl phosphate for acid phosphatase were purchased from SRL, India. ATP for Na^+/K^+ ATPase was purchased from Sigma Chemical Co., USA.

Substrates of marker BBM enzymes :

P-Nitrophenyl phosphate for alkaline phosphatase was purchased from SRL, India and g-glutamyl p-nitroanilide for g-glutamyl transpeptidase was purchased from Sigma Chemical Co., USA.

Radio Chemicals :

Tritiated glucose and proline (^{3}H-Glc & ^{3}H-Pro) and radioactive phosphate (^{32}Pi) were purchased from BARC,

India.

Miscellaneous :

The chemicals used were of finest quality commercially available and their sources are indicated against them. Glass double distilled water was used in all experiments.

CHEMICAL	SOURCE
Acetic acid	E.Merck, India
Ammonium molybdate	Glaxo, India
Bovine serum albumin	Sisco, India
Calcium carbonate	Qualigens, India
Casien	CDH, India
Cholesterol	J.T. Baker Chemical Co., India
Choline chloride	Loba, India
Cocktail-T (Scintillation liquid)	Sisco, India
Copper sulphate ($CuSO_4$)	BDH, India
Creatinine	BDH, India
Cysteine hydrochloride	Sigma Chemical Co., USA
Di-potassium hydrogen ortho-phosphate	Qualigens, India
Di-sodium hydrogen orthophosphate	Qualigens, India
Ferric chloride ($FeCl_3$)	Ranbaxy Lab., India
Ferrous sulphate ($FeSO_4$)	Ranbaxy Lab., India

Folin's Phenol Reagent	Loba Chemical Co., India
Glycine	E. Merck, India
Glycyl-glycine	Loba Chemical Co., India
N-2-Hydroxyethyl-piperazine N-2-ethane sulphonic acid (HEPES)	Sigma Chemical Co., USA
Hydrochloric acid (HCl)	E. Merck, India
Magnesium chloride ($MgCl_2$)	Qualigens, India
Magnesium sulphate ($MgSO_4$)	Sarabhai Chemicals, India
Nicotinamide adenine dinucleotide phosphate (NADP)	SRL, India
Nicotinamide adenine dinucleotide reduced (NADH)	SRL, India
p-Nitroanilide	Sigma Chemical Co., USA
p-Nitrophenol	Loba Chemical Co., India
Ouabain	Sigma Chemical Co., USA
Pentobarbital sodium	Sigma Chemical Co., USA
Perchloric acid	Qualigens, India
Picric acid	Qualigens, India
Potassium chloride	Glaxo, India
Potassium dihydrogen ortho-phosphate	Qualigens, India
Sodium acetate	Glaxo, India
Sodium carbonate (Na_2CO_3)	Qualigens, India
Sodium chloride (NaCl)	E. Merck, India

Sodium dihydrogen orthophosphate	Qualigens, India
Sodium hydroxide (NaOH)	Qualigens, India
Sodium lauryl sulphate (SDS)	CDH, India
Sodium potassium tartarate	Qualigens, India
Sucrose	CDH, India
Sulfuric acid (H_2SO_4)	Qualigens, India
Trichloroacetic acid (TCA)	Loba Chemical Co., India
Tri-iodo thyronine (T_3)	Sigma Chemical Co., USA
Tris-(Hydroxymethyl)amino-methane (Tris-base)	Sigma Chemical Co., USA
Vegetable oil	Anand Milk Producers Union Ltd., India
Vitamin mixture	Glaxo, India

1. Animal Protocol

Adult, male Wistar rats (Experimental Animal Facility Centre, AIIMS, New Delhi, India) weighing 175-250 gm were stabilized for a week prior to the experiment on standard pellet rat diet (Amrut Laboratories, Pune, India) and allowed free access to water. On the day of the experiment, they were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (50 mg/kg body wt.). The abdomen was opened by a left flank incision and the left renal artery was carefully separated from the renal vein. Ischemia was produced by occluding the renal artery with non-traumatic stainless steel arterial clamps (Asian Surgicals, Hyderabad, India) for the desired time intervals as described in "Results". After occlusion of the renal artery, the abdominal viscera were covered with 0.9% buffered NaCl (5 mM Tris-HEPES pH 7.4) soaked gauze. During renal artery occlusion, the ischemic kidney blanched immediately, followed by a colour change to dark violet. With reflow, the kidney resumed its normal colour usually within 1 min. Whenever required, the right kidney was used as contralateral for comparative studies. Sham operated rats were subjected to same surgical procedures described, except that the renal artery was not clamped. In reflow

experiments, ischemia was produced for the desired time as above. The blood flow was resumed to the kidney by removing the clamp for different time intervals (A 2 min. blood reflow was used as a baseline for observing the effect of ischemia). Two milliliters of blood was withdrawn from left jugular vein for analysis of serum parameters before harvesting the kidneys for further analyses.

2. Analysis of Serum Parameters

The serum samples were deproteinated with 3% TCA in the ratio of 1:3. After an incubation for 10 min at room temperature the samples were centrifuged at 4000 rpm (Remi Centrifuge, India) for 10 min. The protein free supernatant was used to quantitate serum creatinine (Cr) and inorganic phosphate (Pi) and the precipitate was saved to quantitate total phospholipids. Cholesterol was determined directly in the serum samples.

(i) Quantitative determination of Creatinine : Creatinine was determined by the method of Levinson and MacFate (180). To 0.5 ml of the deproteinated serum supernatent 250 ul of 10% NaOH and 0.5 ml of saturated picric acid were added and incubated for exactly 20 min at room temperature. A calibration curve was simultaneously

prepared using a known concentration of creatinine solution ranging between 2.0-5.0 ug. The samples and the standards were read at 520 nm in DU-40 spectrophotometer (Beckmann, USA) against a reagent blank.

(ii) **Determination of Inorganic Phosphate** : The inorganic phosphate (Pi) was measured in protein free (TCA precipitated) serum supernatent by the method of Tausky and Shorr (181). The serum supernatent (1 ml) was diluted to 3 ml with glass distilled water and 2 ml of $FeSO_4$ reagent (5 gm $FeSO_4$ was dissolved in 10 ml 10% (w/v) ammonium molybdate in 10 N H_2SO_4 and was diluted to 100 ml with glass distilled water) was added. A calibration curve was prepared simultaneously with test samples using the known concentration of KH_2PO_4 (0.018 umoles to 0.28 umoles). The blue colour obtained was read at 820 nm after 20 min incubation at room temperature in Spectronic 20 spectrophotometer (Bausch and Lomb) against a reagent blank.

iii) **Quantitative determination of phospholipids** : Phospholipids are determined in the serum TCA- precipitates by the method of Bartlet (182) as modified by Marinetti (183). The precipitates were digested with 1 ml 70%

perchloric acid on an electric digestion unit. On cooling to room temperature 3 ml of glass ditilled water was added. The phosphate (inorganic) released was estimated by adding 2 ml of $FeSO_4$ reagent by the method of Tausky and Shorr (181) as described above in Pi estimation.The phospholipid values were obtained after multiplying the phospholipid phosphorus by a factor of 25.

iv) **Quantitative Determination of Cholesterol** : Cholesterol was determined by the method of Zlatkis et al (184). To 3 ml acetic acid, 30 ul serum sample was added. To this, 2 ml of $FeCl_3$ reagent (prepared by diluting 1 ml of 10% $FeCl_3$ (w/v) in glacial acetic acid to 100 ml of concentrated H_2SO_4) was added carefully from the side to allow the formation of a brown ring. The samples were shaken thoroughly, cooled and colour density was read in a Baush and Lomb Spectronic 20 spectrophotometer at 560 nm against a reagent blank. A calibration curve was simultaneously prepared using known concentration (0.02 to 0.2 mg) of cholesterol.

3. **Preparation of Kidney Homogenates for the Determination of Metabolic Enzymes**

After completion of the time of ischemia and/or reflow

the kidneys were removed, decapsulated and kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris-HEPES, pH 7.4). The cortical (whole cortex (WC) or superficial (SC) and juxtamedullary (JM) cortex) and medullary regions were carefully separated as described below (165) (Fig. 1), and homogenized in 0.1 M Tris-HCl buffer pH 7.5 by a glass-teflon homogenizer at 4°C to make a 15% homogenate. The homogenate was centrifuged at 800 xg at 4°C for 10 min in Beckman T2-MI high speed refrigerated centrifuge to remove the cell debris. The supernatent was used for enzyme analysis (see "Method" for detail).

4. Brush Border membrane (BBM) Preparation

After the completion of time of ischemia and ischemia and/or reflow the kidneys were removed, decapsulated and kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris-HEPES, pH 7.5). The cortical tissues were carefully separated for BBM preparation as whole cortex (WC), superficial cortex (SC) and juxtamedullary cortex (JMC) as described by Yusufi et al (165). In brief, the cortical part of the kidney was cut into two halves and the whole cortex (WC) was separated from medullary and papillary portions. For the preparation of BBMV-SC and BBMV-JMC, the cortical

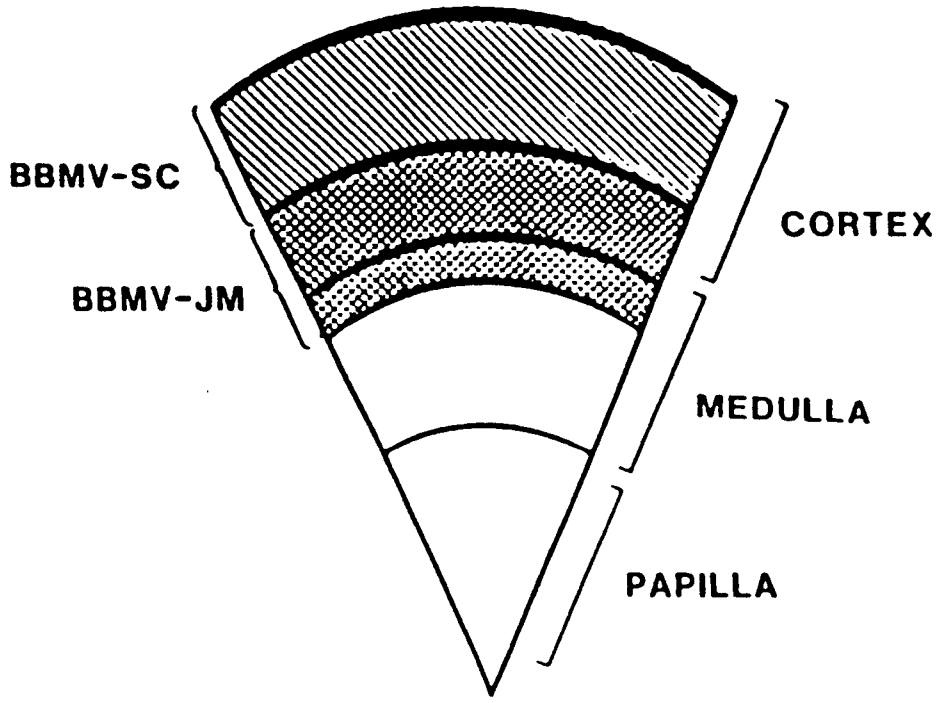


Fig. 1. Anatomical parts of the kidney - A longitudinal view.

portion was cut in the middle of the cortical thickness (Fig. 1) between the kidney surface and corticomedullary junction. The juxtamedullary part of the cortex was carefully separated from superficial cortex. The outer cortical zone, superficial cortical tissue (SC) "SC-tissue" was used as a starting material for BBMV-SC preparation. Since, in rat kidney, straight portions of the proximal tubules dip beyond the cortico-medullary junction, the outermost layer of adjacent medulla (outer stripe of outer medulla) was also included to juxtamedullary cortical tissue i.e. "JMC-tissue".

The BBM vesicles (BBMV) were prepared by the method of Schimtz et al (185) using $MgCl_2$ for precipitation of membranes other than BBM as described by Yusufi and Dousa (48) and as outlined in the schematic diagram (Fig. 2). In each experiment tissues from three to six animals (control and experimental) were pooled to obtain a sufficient amount of starting material. All the steps involved were strictly carried out at 0-4°C unless otherwise specified.

a) The cortical tissue for the preparation of BBMV was homogenized in a buffered solution containing 50 mM mannitol, 5 mM Tris base/HEPES, pH 7.0 (5 ml/g tissue)

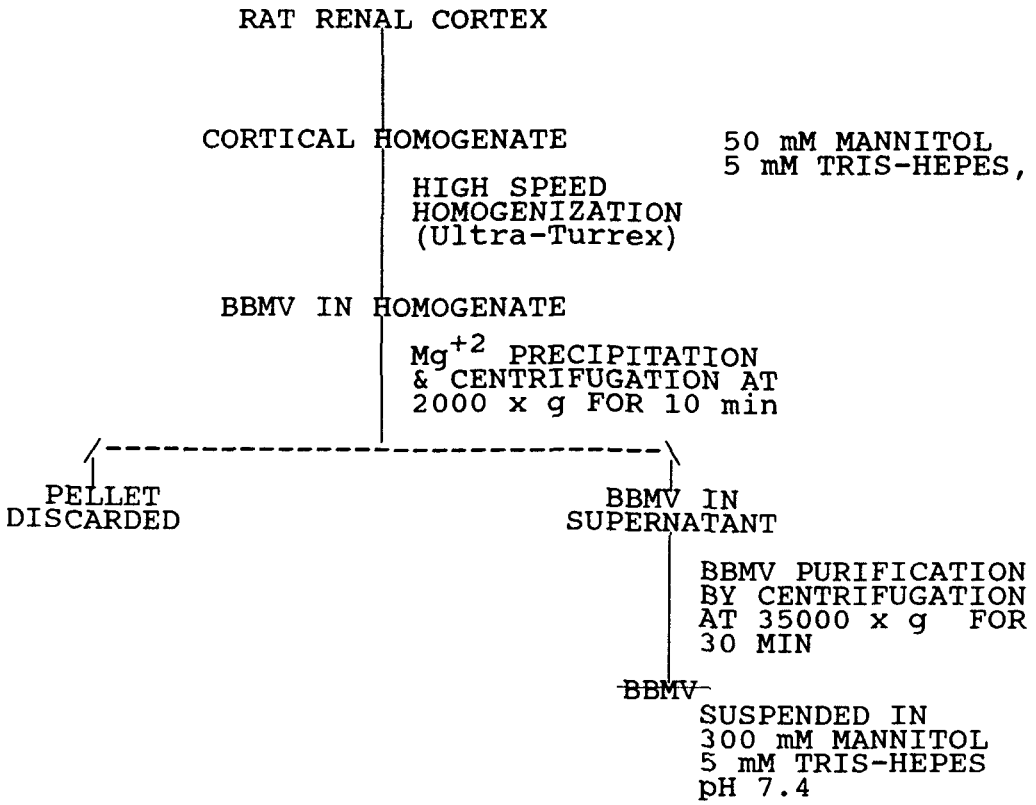


Fig. 2 : Schematic representation of BBMV preparation from rat renal cortex.

with four complete passes by Potter-Elvejhem homogenizer.

b) The homogenate was diluted with the above solution (20 ml/g tissue) followed by high speed homogenization (Ultra-Turrex T25, IKA-Laportechnik) with three pulses of 30s each with 30s interval in between each pulse. Aliquouts of cortical homogenates were saved and quickly frozen for further analysis.

c) 1 M $MgCl_2$ was added to the homogenate (final conc. 10 mM) and was kept for 20 min with intermittent shaking.

d) The homogenate was then centrifuged at 2000 xg (4000 rpm) for 10 min in a Beckman J2-21 refrigerated centrifuge using JA-17 rotor.

e) The pellet was discarded and the supernatant was recentrifuged at 35000 xg (17000 rpm) for 30 min.

f) The pellet thus obtained was resuspended in a solution containing 300 mM mannitol, 5 mM Tris base/HEPES pH 7.5 with four passes by a loose fitting Dounce homogenizer (Wheaton, USA) and centrifuged at 35000 xg (17000 rpm) for 20 min in 15 ml cortex tube using JA-20 rotor.

g) The white outer portion of the fluffy pellet was resuspended carefully in a small volume of buffered 300 mM mannitol, leaving the dark brown centre of the pellet undisturbed (mitochondrial contamination).

h) The steps f and g were repeated twice and the final pellet was resuspended in buffered 300 mM mannitol.

The suspension thus obtained was homogenized by hand held Douncer or passed through a needle no. 21. The membrane suspension was quickly frozen in small aliquots and used for enzyme analysis while aliquots of freshly prepared membranes were used for transport studies.

5. Enzyme Assay

All enzymes were assayed at zero order kinetics unless otherwise specified. The activities of each enzyme from various comparing groups were determined simultaneously under similar conditions by using same solutions to avoid day to day experimental variations. One unit of the enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 umole of product per min or hr under the specified experimental conditions. Specific activity is defined as the enzyme unit per mg of enzyme protein.

(a) **Assay of carbohydrate metabolism enzymes** : The assays were carried out measuring the extinction changes in Calbiometer (Calbiochem, U.S.A.) fixed for 340 nm in a final volume of 3.0 ml at room temperature (28-30°C). The net reaction rate was measured by difference of the extinction values obtained from addition of the substrate and for actual enzymic reaction following the addition of substrate.

(i) **Lactate dehydrogenase (L-Lactate : NAD oxidoreductase; LDH; E.C. 1.1.1.27)** : The activity of LDH was measured by the method of Kornberg (186). The reaction mixture in a total volume of 3 ml contained Tris-HCl buffer pH 7.4, 150 umoles; $MgCl_2$ 10 umoles; sodium pyruvate 5 umoles; NADH 0.24 umoles and 4.0-6.0 ugm protein. The activity was measured pyruvate dependent NADH oxidation to NAD^+ for 5 min at 340 nm (ϵ = 6.22 $mM^{-1}cm^{-1}$) in Calbiometer (Calbiochem, U.S.A.).

(ii) **Malate dehydrogenase (L-malate : NAD oxidoreductase; MDH; E.C. 1.1.1.37)** : The activity of MDH was measured according to the method of Meyer et al (187). The reaction mixture in a total volume of 3 ml contained Tris-HCl buffer pH 7.4, 100 umoles; oxaloacetate (OAA, pH neutralized to 7.4) 2.5 umoles; NADH 0.24 umoles and

2.0-3.0 ugm protein. The activity was measured as OAA dependent NADH oxidation to NAD^+ for 5 min at 340 nm (ϵ = 6.22 $mM^{-1}cm^{-1}$) in Calbiometer (Calbiochem, USA).

(iii) **Malic enzyme (L-malate : NADP oxidoreductase; ME; E.C. 1.1.1.40)** : It was assayed according to the method of Ochoa et al (188). The reaction mixture in a total volume of 3 ml contained Tris-HCl buffer, pH 7.4, 100 umoles; $MnCl_2$ 10 umoles; L-malic acid (pH neutralized to 7.4) 5 umoles; $NADP^+$ 0.24 umoles; and 0.6-1.2 mg protein. The activity was measured by monitoring the malic acid dependent reduction of $NADP^+$ to NADPH for 5 min at 340 nm (ϵ = 6.22 $mM^{-1}cm^{-1}$) in Calbiometer (Calbiochem, USA),.

(iv) **Glucose-6-phosphate dehydrogenase (D-Glucose-6-phosphate NADP oxidoreductase; G6PDH; E.C. 1.1.1.49)** : G6PDH was assayed according to the method of Shonk and Boxer (189). The reaction mixture in a total volume of 3 ml contained Tris-HCl buffer pH 7.4, 150 umoles; $MgCl_2$ 10 umoles; Glucose-6-phosphate 5 umoles; $NADP^+$ 0.24 umoles; and 0.6-1.2 mg protein. The activity was measured by monitoring the G-6-P dependent reduction of $NADP^+$ to NADPH for 5 min at 340 nm (ϵ = 6.22 $mM^{-1}cm^{-1}$)

in Calbiometer (Calbiochem, USA)

(v) **Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; G6Pase; E.C. 3.1.3.9)** : It was assayed according to the method of Shull et al (190). The reaction mixture in a total volume of 1.5 ml contained Tris-HCl buffer pH 7.4, 50 umoles; $MgCl_2$ 10 umoles; Glucose-6-phosphate 10 umoles and 2-3 mg protein. The reaction was carried out at 37°C and stopped with 1 ml of 10% TCA after 60 min. The samples were centrifuged at 4000 rpm (Remi Centrifuge, India) and phosphorus was estimated in the protein free supernatent by the method of Tausky and Shorr (181).

(vi) **Fructose-1,6-bisphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase; FBPase; E.C. 3.1.3.11)** : It was assayed according to the method of Freedland and Harper (191). The reaction mixture in a total volume of 1.5 ml Tris-HCl pH 8.4, 50 umoles; $MgCl_2$ 10 umoles; Cysteine-HCl 12 umoles; F-1,6-DP 10 umoles; 0.6-0.8 mg protein. The reaction was carried out at 37°C and was stopped with 1.0 ml 10% TCA after 60 min. The samples were centrifuged at 4000 x rpm (Remi Centrifuge, India) and the phosphate released was estimated in protein free

supernatent by the method of Tausky and Shorr (181).

(vii) **Na^+-K^+ ATPase (E.C. 3.6.1.3)** : The activity of Na^+-K^+ ATPase was assayed as described by Szczepanska-Konkel et al (192). Enzyme preparations were incubated for 15 min at 37°C in a medium containing in a final volume of 1.5 ml (Final concentration) 4.8 mM ATP; 120 mM NaCl; 24 mM KCl, 7.2 mM $MgSO_4$ and 48 mM Tris-HCl pH 7.6. The ATPase activity without the addition of Ouabain is referred to as "Mg-ATPase". The difference in Mg-ATPase activity assayed without or with 1.2 mM ouabain is taken as a measure of Na^+-K^+ ATPase (193). The reaction was terminated by the addition of 0.3 ml 30% TCA and the Pi released was determined by the method of Tausky and Shorr (181).

(b) **Assay of marker enzymes of brush border membrane** : The enzymes were assayed simultaneously in CH and BBMV under similar conditons by using same solutions to avoid day to day experimental variations. Aliquots of CH and BBMV were diluted with 10 mM Tris-HCl buffer, pH 7.5 to obtain suitable enzyme protein concentration.

(i) **Alkaline phosphatase (AlkPase, E.C. 3.1.3.1)** : The

activity of AlkPase in CH and BBM was determined according to the method of Shah et al (194) as described by Kempson et al (195). The reaction mixture contained 1.4 ml assay buffer (55 mM glycine; 36 mM NaCl; and 45 mM NaOH, pH 10.5) and 100 ul enzyme protein (10-25 ug for CH and 4-8 ug for BBM). The reaction was started by adding 15 ul p-nitrophenyl-phosphate (final concentration 5.8 mM in 1.5 ml) and incubated at 30°C for the required time (5-20 min). The reaction was stopped by adding 50 ul 5 N NaOH. A calibration curve was prepared simultaneously by using known concentrations of p-nitrophenol (0.01-0.2 umoles). The colour was read at 405 nm in Spectronic-21 spectrophotometer (Baush and Lomb) against a reagent blank.

(ii) **g-Glutamyl transferase (GGTase, E.C. 2.3.2.2)** : The activity of g-Glutamyl transferase (GGTase) in CH and BBM was determined according to the method of Glossmann and Neville (196) as described by Kempson et al (197). The reaction was started by adding 100 ul enzyme protein (15-20 ug for CH; 2-5 ug for BBM) to 1.9 ml substrate buffer (20 mM $MgCl_2$, 2 mM g-glutamyl p-

nitroanilide, 4 mM glycylglycine, 100 mM Tris-base pH 8.2) and incubated in a shaking water bath maintained at 37°C for the required time (3-15 min). The reaction was stopped by adding 100 ul 15 M acetic acid. A calibration curve of known concentration of standard p-nitroaniline (0.025-0.2 umoles) was prepared simultaneously. The yellow colour developed was read at 405 nm in Spectronic-21 spectrophotometer (Bausch & Lomb) against a reagent blank.

(iii) **Na^+-K^+ ATPase** : The activity of Na^+-K^+ ATPase in CH and BBM was assayed as described above.

(iv) **Acid phosphatase (APase, E.C. 3.1.3.2)** : The activity of acid phosphatase (APase) was determined in CH and BBM by the method of Verjee (198). The reaction mixture contained 2.4 ml acetate buffer (0.05 M sodium acetate, pH 4.5) and 100 ul enzyme (35-40 ug both in CH & BBM). The reaction was started by the addition of 0.5 ml p-nitrophenyl phosphate (final conc. 0.8 mM in 3 ml) and incubated for 15 min at 30°C. The reaction was stopped by adding 2 ml 2 N NaOH. A calibration curve of known concentrations (0.05-0.4 umoles) of p-nitrophenol was prepared simultaneously. The yellow colour

developed was read at 405 nm in Spectronic-20 spectrophotometer against a reagent blank.

6. Protein Estimation

Protein was measured by the method of Lowry et al (199) with minor modification as described previously by Yusufi et al (200). The sample was made to 0.8 ml with 0.5% SDS. In timed sequence 2.0 ml alkaline copper reagent was added and exactly after 10 min of incubation at room temperature (RT) 0.2 ml Folin's reagent (1 N) was added with brisk shaking and incubated for 30 min at RT. A calibration curve of standard BSA (5-8 ug) was prepared simultaneously. The blue colour obtained was read at 660 nm against a reagent blank in Spectronic-20 spectrophotometer (Bausch & Lomb).

7. Transport

Measurement of phosphate (^{32}Pi), L-[^{3}H]- proline and D-[^{3}H]-Glucose (BARC, India) uptakes in brush border membrane vesicles was carried at 25°C by rapid filteration technique as described by Yusufi et al (165) either in the presence or absence of Na-gradient. Uptake was initiated by addition of 30 ul incubation medium (NaCl/KCl 100 mM, 5 mM Tris-HEPES, pH 7.5, 5mM K_2HPO_4/ 0.025 mM proline/0.05 mM D-glucose and radioactive substrate ^{32}Pi/L-[^{3}H]-pro/D-[^{3}H]-glu) to 15 ul

BBM suspension (50-100 ug protein) and incubated for desired time intervals (see "Results"). At the end of incubation period the uptake was stopped by rapid addition of 3 ml ice-cold stop solution (delivered by a Cornwall syringe type pipette) containing 135 mM NaCl, 5 mM Tris-HEPES with (for Pi uptake) or without (for glucose and proline uptake) 10 mM sodium arsenate, pH 7.5 and filtered immediately through 0.45 um DAWP millipore filters and washed 3 times with the same ice-cold stop solution. Correction for non-specific binding to filters was made by substracting from all data the value of the corresponding blank obtained by filteration of the incubation medium without vesicles. The radioactivity of the dried filters was measured by liquid scintillation counting (Rackbeta, LKB, Wallac) with 10 ml scintillation fluid ("Cocktail-T", SRL, India).

8. Preparation of Low Phosphorus Diet (LPD)

The LPD containing 0.07% phosphates was prepared in the laboratory, the composition of which is similar to the commercially available low phosphate diet of ICN Pharmaceuticals Inc., Cleveland, Ohio. The LPD contained (in gm%) sucrose 66, casein 20, salt mix (1.02% NaCl; 0.53% KCl; 0.305% $MgSO_4$; 0.103% iron citrate; 1.02% $CaCO_3$ and 0.014%

cupric sulfate) 4, vitamin mixture (Glaxo, India) 0.4 and vegetable oil 10. The normal phosphorus diet (NPD) was prepared by supplementing with a mixture of sodium and potassium phosphates (ratio of monobasic : dibasic salt was 1:4) to a final content of 0.7% (w/w) phosphorus, as described by Kempson et al (201).

9. Preparation of Thyroxine (T_3)

4.37 mg T_3 was dissolved in 10 ml, NaOH (10 mM). 10 ml Tris (5 mM) in 1.8% NaCl was added to the T_3 solution. The pH was slowly adjusted to 9.4±0.1 with 0.1 N HCl. The solution was diluted to 25 ml with a mixture of above solution (10 ml, 10 mM NaOH, 10 ml 5 mM Tris in 1.8% NaCl pH 9.4±0.1) which was used as vehicle for control rats.

10. Animal Protocol For LPD And T_3 Treatment

Separate groups of animals were placed simultaneously on the different dietary regimens and through out the experiment all groups were studied in parallel (Fig. 39). The animals were first stabilized on NPD for seven days. After day 7, the animals were divided into four groups (12 rats in each group) and the allocation of animals was adjusted so that at this time the groups did not differ in body weight or urinary Pi. The rats were than fed with

specified diets for seven days. Group I received NPD + 1 ml injection of vehicle, group II received NPD + 1 ml T_3 (final conc. 100 ug/100 g body weight) injection, group III received LPD + 1 ml injection of vehicle, group IV received LPD + 1 ml T_3 (final conc. 100 ug/100 g body weight) injection intraperitoneally.

On the day of experiment (day 15), the rats were anaesthetized by an intrape-ritoneal injection of sodium pentobarbital (50 mg/Kg body wt.). Ischemia was produced for 30 min as described elsewhere. After the time of ischemia, blood was allowed to reflow for either 15 or 60 min before harvesting the kidneys for BBMV preparation. BBMVs from whole cortex were prepared as described above. Transport of ^{32}Pi was studied by rapid filteration technique as described earlier in "Method".

Statistical Analysis : Unless specified, all experiments were repeated atleast three or four times to document reproducibility. All data are expressed as Mean±SEM. When appropriate, statistical evaluation was used and conducted employing Student's t-test.

RESULTS & DISCUSSION
PART I

RESULTS - I

General

Renal ischemia in rats was produced by occlusion of left renal artery for different time periods as indicated in the "Results" followed by a brief reflow of blood for 2 min to clear the kidney tissue before harvesting the kidney for subsequent analysis and this procedure was applied throughout the study and was considered as the base-line for observing the damage caused by ischemia on various parameters.

In one set of experiments, ischemia was produced by clamping the left renal artery for 15,30,45 and/or 60 min. The damage caused by the ischemia was monitored by serum creatinine levels. The results summarized in Table 1 indicate that after 15 min of ischemia serum creatinine was significantly increased (25%) in ischemic rats compared to sham-operated control rats. Further increase in serum creatinine was observed when ischemia was produced for 30 min (56%) and for a longer duration i.e. for 60 min (80%) as well. Similar to creatinine the serum levels of inorganic Pi, phospholipids and cholesterol (Chol) were also increased significantly, however, differentially with respect to the duration of ischemia (Table 1).

TABLE-1: EFFECT OF 15-60 min ISCHEMIA ON SERUM PARAMETERS

Time of Ischemia	Creatinine (ug/ml serum)	Inorganic Phosphate (umol/ml serum)	Phospholipids (mg/ml serum)	Cholesterol (mg/ml serum)
0 min (n = 12)	22.95±0.92[a]	1.53±0.03	0.567±0.02	1.88±0.03
15 min (n = 15)	28.58±0.65[b]	1.76±0.06[b]	0.754±0.03[b]	2.08±0.03[b]
30 min (n = 15)	35.90±0.57[b]	2.13±0.06[b]	1.084±0.02[b]	2.29±0.03[b]
60 min (n = 15)	41.29±1.01[b]	2.63±0.05[b]	1.616±0.08[b]	2.65±0.05[b]

[a]Values are Mean±SEM for n samples indicated in parentheses.

[b]Significantly different from Control (0 min) $p < 0.01$ by group t-test.

Table 2: EFFECT OF 15 min ISCHEMIA AND REFLOW ON SERUM PARAMETERS

TIME OF REFLOW	CREATININE ug/ml serum	INORGANIC PHOSPHATE umol/ml serum	PHOSPHOLIPIDS ug/ml serum	CHOLESTEROL mg/ml serum
2 min	50.19±4.28[a]	2.62±0.11	249.67±36.05	3.28±0.11
15 min	49.61±6.74	2.24±0.035[b]	322.00±43.77[b]	3.09±0.086[b]
60 min	34.15±3.65[b]	2.33±0.16[b]	214.67±42.64[c]	2.58±0.11[b]
120 min	34.67±4.28[b]	2.18±0.07[b]	218.09±35.23[c]	2.44±0.06[b]

[a]Values are Mean±SEM n=6 samples in each group.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

Control values were 31.30, 0.98, 150 and 2.37 respectively for Cr, Pi, PL and Chol.

Table 3: EFFECT OF 30 min ISCHEMIA AND REFLOW ON SERUM PARAMETERS

TIME OF REFLOW	CREATININE ug/ml serum	INORGANIC PHOSPHATE umol/ml serum	PHOSPHOLIPIDS ug/ml serum	CHOLESTEROL mg/ml serum
2 min	56.08±3.76	2.70±0.17	292.27±17.87	3.31±0.098
15 min	42.86±6.21	2.40±0.14[b]	334.60±18.20[b]	3.24±0.098[b]
60 min	41.53±5.72[b]	2.02±0.21[b]	220.73±43.06[c]	2.79±0.16[b]
120 min	42.69±6.11[b]	1.77±0.041[b]	173.13±27.31[c]	3.10±0.07[b]

[a]Values are Mean±SEM for n=6 samples in each group.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

Control values were 31.30, 0.98, 150 and 2.37 respectively for Cr, Pi, PL and Chol.

When the blood was allowed to reflow in the ischemic rats for different time periods (15 min - 120 min), a reversal of the effect due to ischemia was observed in serum Cr, Pi and Chol levels, both in 15 min and 30 min ischemic rats. The above serum parameters tended to return back towards normal values after 120 min of reflow (Table 2 & 3).

Effect of ischemia on the activities of BBM-marker enzymes

The kidneys were harvested from the animals and the brush border membranes were isolated from whole cortex. The effect of ischemia was determined on BBM-marker enzymes and enzymes of other organelles/memb-ranes. The specific activities of both alkaline phosphatase (AlkPase) and g-glutamyl transferase (BBM-marker enzymes) were significantly but differentially decreased in BBMV(s) of ischemic rats compared to control rats (Fig. 3) after 15 min of ischemia. Further decline in the activities was observed when ischemia was produced for 30 min or 60 min duration. The maximum decrease was obtained after 60 min of ischemia indicating a greater damage to the proximal tubular membranes upon prolonged ischemia (Table 4 & 5).

TABLE-4: EFFECT OF 15-60 min ISCHEMIA ON THE ACTIVITY OF ALKALINE PHOSPHATASE (AlkPase) IN CH AND BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Ischemia	BBMV	CH
0 min	77.15±6.50[a]	7.00±0.48
15 min	58.85±5.40[b]	7.23±0.35
30 min	44.31±3.47[b]	6.91±0.54
60 min	41.10±8.50[b]	6.45±0.32
Contralateral		
15 min	67.20±5.11	7.25±0.56
30 min	67.71±5.23	6.94±0.48
60 min	69.53±10.49	6.38±0.19

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

TABLE-5: EFFECT OF 15-60 min ISCHEMIA ON THE ACTIVITY OF g-GLUTAMYL TRANSFERASE (GGTase) IN CH AND BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Ischemia	BBMV	CH
0 min	400.50±32.43[a]	31.26±2.08
15 min	323.13±46.37	32.59±1.19
30 min	257.96±35.85[b]	37.24±2.04
60 min	212.70±22.24[b]	28.99±1.70
Contralateral		
15 min	356.81±68.33	32.39±3.41
30 min	346.63±61.62	32.23±1.28
60 min	430.12±33.31	31.91±3.46

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

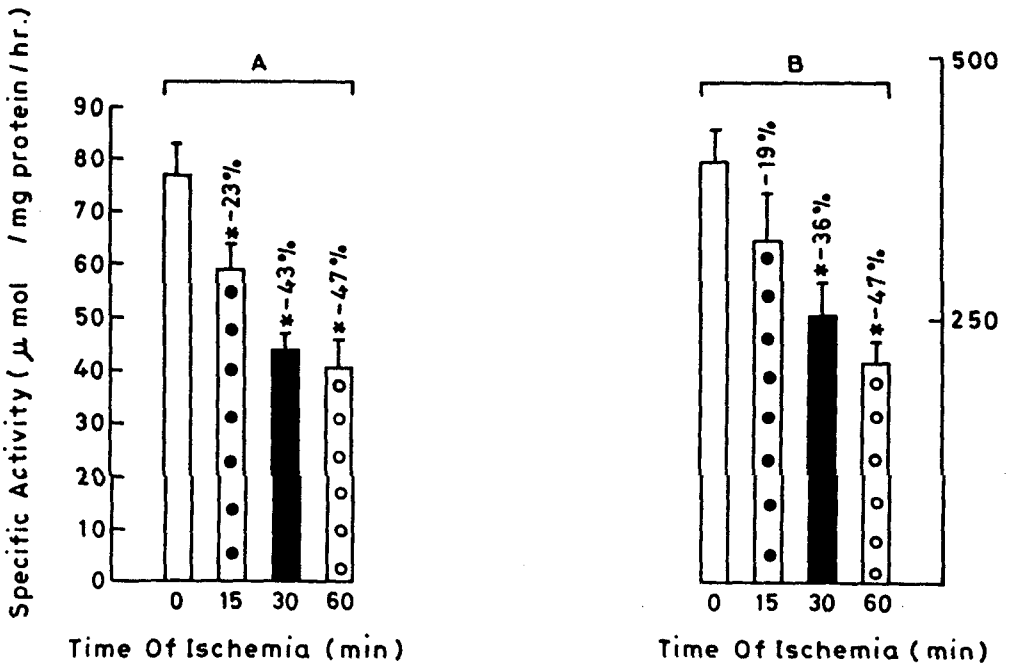


Fig.3: Specific Activities of (A) AlkPase and (B) GGTase in BBMV(s) from whole Cortex after ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control(0 min) $P < 0.05$ or higher degree by group t-test.

To determine the effect of reflow the rats were allowed to perfuse with the blood for various time period, as shown in Fig. 4. Initial reflow of blood for 15 min caused further decrease in the activities, of both AlkPase (Fig. 4a) and GGTase (Fig. 4b). The decrease in the enzyme activity was significantly greater in 60 min compared to 30 min ischemic rats (54% vs 36% for AlkPase; 70% vs 30% for GGTase) and the activity of GGTase was more affected than that of AlkPase (Fig. 4a; 4b). Further, reflow for upto 120 min showed a sharp reversal in the enzyme activities, and both AlkPase and GGTase activities were restored upto 86 and 92% of the control values in 30 min ischemic rats. However, the activities were recovered to a lesser extent (50-60% of the control values) in 60 min ischemic rats indicating a greater damage to the membrane components after prolonged ischemia, which could not be restored completely even after 120 min blood reflow (Fig. 4a and 4b). The activities of both the enzymes in the cortical homogenate (CH) in control, contralateral and ischemic kidneys were not significantly different (Table 6-9).

When the activities (expressed as total enzyme units) of the enzymes were analyzed, total activities of the

TABLE-6: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF ALKALINE PHOSPHATASE (AlkPase) IN CH AND BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Reflow	BBMV	CH
Control	177.43±2.36[a]	18.43±0.96
2 min	140.91±0.95[b]	14.86±0.22
15 min	112.79±0.74[b]	15.05±0.30
60 min	140.35±0.47[b,c]	14.51±0.82
120 min	153.82±2.13[b,c]	13.68±0.44

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min RF group $p < 0.05$ or greater degree by group t-test.

TABLE-7: EFFECT OF 60 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF ALKALINE PHOSPHATASE (AlkPase) IN CH AND BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Reflow	BBMV	CH
Control	177.43±1.50[a]	18.07±0.13
2 min	126.95±0.53[b]	16.42±0.12
15 min	81.14±0.68[b]	15.84±0.21
120 min	108.09±0.88[b,c]	17.61±0.22

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min RF $p < 0.05$ or greater degree by group t-test.

TABLE-8: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF g-GLUTAMYL TRANSFERASE (GGTase) IN CH AND BBMV ISOLATED FROM WHOLE CORTEX.

Time of Reflow	BBMV	CH
Control	246.73±1.93[a]	23.89±0.19
2 min	190.35±2.02[b]	29.89±0.25
15 min	172.46±2.95[b]	31.06±0.41
60 min	202.59±3.50[b,c]	28.91±0.92
120 min	226.09±3.57[b,c]	22.25±0.44[c]

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min RF $p < 0.05$ or greater degree by group t-test.

TABLE-9: EFFECT OF 60 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF g-GLUTAMYL TRANSFERASE (GGTase) IN CH AND BBMV ISOLATED FROM WHOLE CORTEX.

Time of Reflow	BBMV	CH
Control	245.17±4.24[a]	24.93±0.21
2 min	84.83±1.22[b]	20.68±0.55
15 min	72.97±1.48[b]	18.55±0.31
120 min	121.15±2.33[b,c]	19.60±0.37[b]

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min RF $p < 0.05$ or greater degree by group t-test.

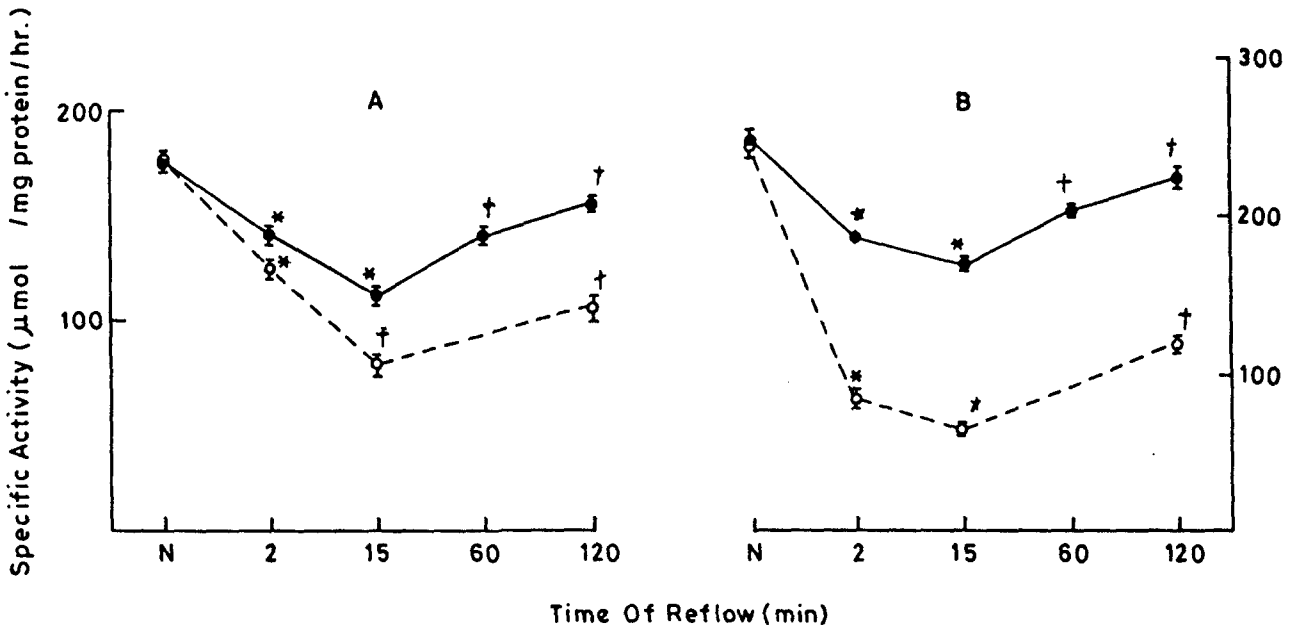


Fig.4: Specific Activities of (A) AlkPase and (B) GGTase in BBMV(s) from whole cortex after 30 min (—●—) and 60 min (--O--) ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p < 0.05$ or higher degree by group t-test.

†Significantly different from 15 min RF group $p < 0.05$ or higher degree by group t-test.

membrane-bound enzymes were declined and the activities in the supernatant (free/enzymes released due to ischemia) were found to be increased in proportion to the duration of the ischemia i.e. the activities of the free-supernatant enzymes were higher after 60 min when compared to 30 min of ischemia (Table 10 & 11). This further supports that the membrane enzyme components are dissociated from the membrane structure due to ischemic damage and became free soluble components (Fig. 5a; 5b).

As morphologic and other studies indicated that the nephrons from the deep cortex or the pars recta are greately damaged by the ischemia, BBM-vesicles were isolated from superficial (BBMV-SC) and juxtamedullary (BBMV-JMC) cortex and the damage caused by the ischemia were extensively studied in subsequent experiments. As shown in Fig. 6 (a, b) the activity of AlkPase was decreased in ischemic rats both in BBMV-SC and BBMV-JMC as compared to respective control or contralateral values. The decrease in the activity was always linearly proportional to the time of the ischemia (Table 12 & 13). Similar results were also obtained for GGTase (Fig. 7 (a & b); Table 14 & 15). However, the decrease in the activity of both the enzymes was greater in

TABLE-10: EFFECT OF ISCHEMIA (30 and 60 min) AND 15 AND 120min REFLOW ON TOTAL ACTIVITY OF BOUND AND FREE AlkPase IN THE CORTICAL HOMOGENATES FROM WHOLE CORTEX.

Time of Reflow	CONTROL			
	BOUND		FREE	
	1569.60±110.98[a]		356.96±13.50	
	30 min Isch.		60 min Isch.	
	Bound	Free	Bound	Free
15 min	945.17±31.73[b]	906.72±98.10[b]	636.19±32.95[b]	1048.32±99.60[b]
120 min	1245.53±48.85[c]	668.32±39.43[c]	740.79±53.33[b]	794.88±69.00[b]

[a] Activities are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b] Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c] Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

TABLE-11: EFFECT OF ISCHEMIA (30 and 60 min) AND 15 AND 120 min REFLOW ON TOTAL ACTIVITY OF BOUND AND FREE GGTase IN CORTICAL HOMOGENATES FROM WHOLE CORTEX.

Time of Reflow	CONTROL			
	BOUND		FREE	
	5903.99±86.30[a]		469.28±13.80	
	30 min Isch.		60 min Isch.	
	Bound	Free	Bound	Free
15 min	3255.12±55.15[b]	823.68±18.97[b]	2376.01±45.80[b]	1208.00±10.98[b]
120 min	4664.15±98.60[c]	527.04±13.98[c]	2597.36±30.90[c]	806.24±14.50[c]

[a]Activities are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

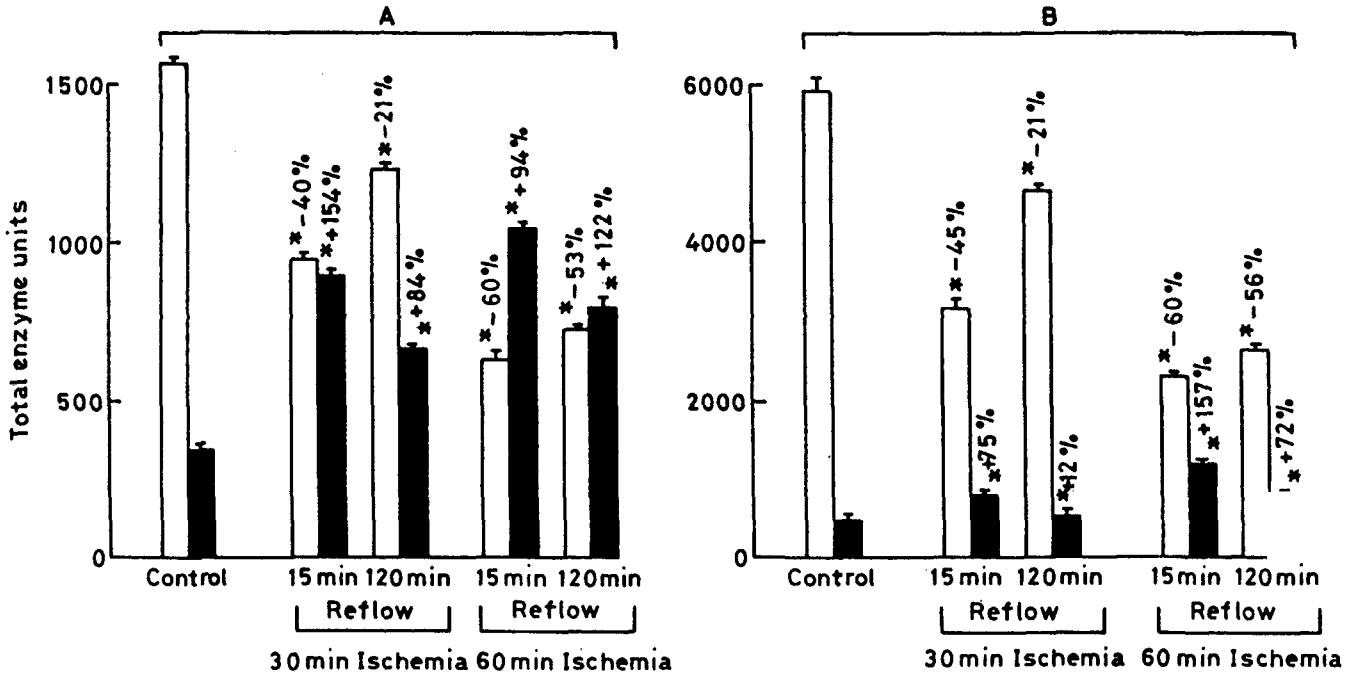


Fig.5: Total Enzyme activities of (A) AlkPase and (B) GGTase, after 30 and 60 min ischemia followed by reflow of 15 and 120 min. (□) bound; (■) free.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p < 0.05$ or higher degree by group t-test.

BBMV-JMC compared to BBMV-SC (Table 12-15). The results further indicate that the decrease in the enzyme activities were mainly due to decrease in the Vmax of the enzyme activities rather than in the Km values (Table 16 & 17; Fig. 8-11). The activities of both the enzymes were not significantly different in CH of control, contralateral or ischemic kidneys (Table 12-15). When the blood was allowed to reflow for different time periods, the activities of both AlkPase (Fig. 12 & 13) and GGTase (Fig. 14 & 15) declined further after 15 min of reflow following ischemia of 15 & 30 min in both BBMV-SC and BBMV-JMC, as was observed in whole cortex. Further reflow for upto 120 min resulted in reversal of decrease in the activities of both AlkPase and GGTase in both SC and JMC (Fig. 12-15). However, the reversal of GGTase was slower in BBMV-JMC than in BBMV-SC indicating greater damage to the BBMV-JMC than BBMV-SC or deep nephrons than the superficial nephrons. The activities of both the enzymes in the contralateral kidneys were not significantly different from sham-operated normal kidneys (Table 18-21). The activities of both the enzymes were not significantly different in CH of control, contralateral or ischemic kidneys (data not shown).

Table 12: EFFECT OF 15-60 min ISCHEMIA ON ALKALINE PHOSPHATASE ACTIVITY IN CH AND BBM ISOLATED FROM SUPERFICIAL CORTEX.

	CH		BBM		
CONTROL	10.26 ± 1.21[a]		100.37 ± 2.19		
	CONTRALATERAL		ISCHEMIA		
	CH	BBM	CH	BBM	%decrease[c]
Ischemia 15 min	11.64±0.79	94.00±1.25	7.19±2.22	66.76±10.90[b]	33.50
Ischemia 30 min	12.79±1.96	87.38±12.17	9.18±2.06	59.40±7.84[b]	40.82
Ischemia 45 min	7.45±0.26	87.20±11.37	9.24±0.88	50.95±4.22[b]	49.24
Ischemia 60 min	9.43±2.52	75.86±10.39	7.02±0.19	40.56±4.13[b]	59.59

[a]Activities (umoles/mg protien/hr) is expressed as Mean ±SEM of three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control value, $p < 0.05$ by group t-test.

[c]Percent decrease from Control values.

Table 13: EFFECT OF 15-60 min ISCHEMIA ON ALKALINE PHOSPHATASE ACTIVITY IN CH AND BBMV(s) ISOLATED FROM JUXTAMEDULLARY CORTEX.

	CH	BBM			
CONTROL	8.55 ± 1.82[a]		109.40 ± 9.81		
	CONTRALATERAL		ISCHEMIA		
	CH	BBM	CH	BBM	%decrease[c]
Ischemia 15 min	10.60±0.38	103.67±2.52	7.28±0.64	58.24±12.16[b]	46.76
Ischemia 30 min	10.77±0.05	97.32±4.54	7.71±2.19	50.44±5.66[b]	53.89
Ischemia 45 min	7.65±0.53	90.61±3.66	7.92±1.08	40.60±5.46[b]	62.89
Ischemia 60 min	11.86±4.20	90.86±0.67	6.78±0.11	35.22±0.59[b]	67.81

[a]Activities (umol/mg protein/hr)are expressed as Mean±SEM of three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control value $p < 0.05$ by group t-test.

[c]Percent decrease from Control values.

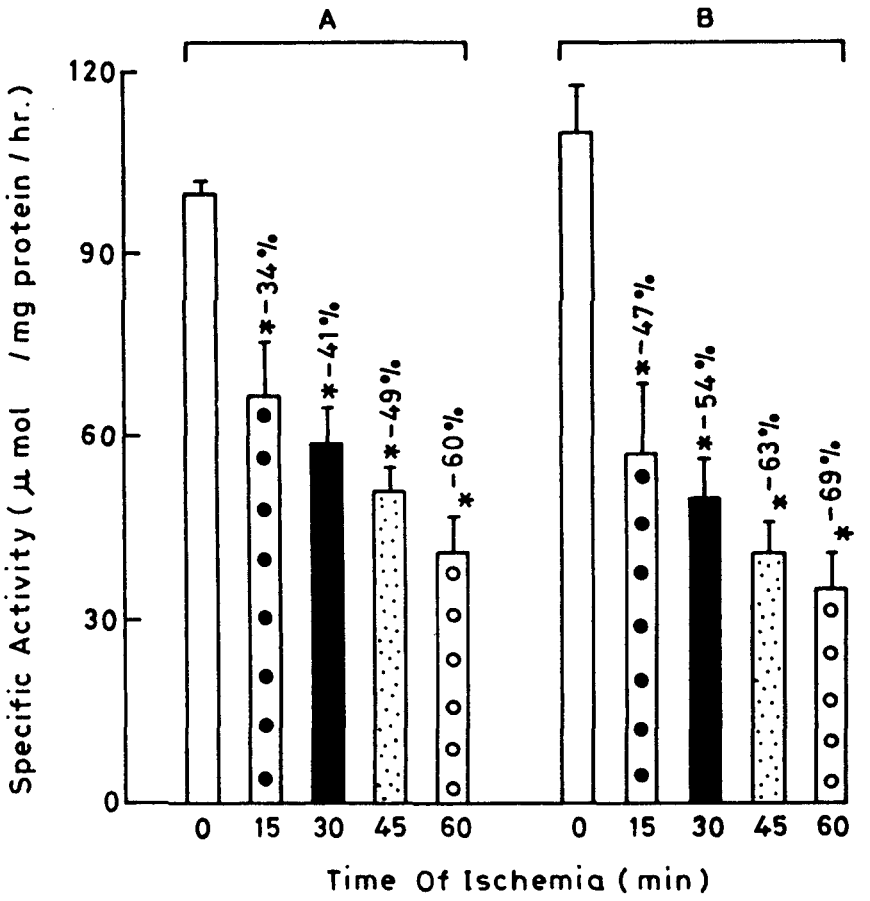


Fig.6: Specific Activity of AlkPase in BBMVs from (A) Superficial cortex (BBMV-SC) and (B) Juxtamedullary cortex (BBMV-JMC) after 15-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (O min) $p < 0.05$ or higher degree by group t-test.

Table 14: EFFECT OF 15-60 min ISCHEMIA ON g-GLUTAMYL TRANSFERASE ACTIVITY IN CH AND BBMV(s) ISOLATED FROM SUPERFICIAL CORTEX.

	CH		BBM		
CONTROL	15.50 ± 2.25[a]		140.21 ± 17.44		
	CONTRALATERAL		ISCHEMIA		
	CH	BBM	CH	BBM	%decrease[c]
Ischemia 15 min	14.37±1.26	130.97±5.31	17.64±4.40	104.81±10.18	25.25
Ischemia 30 min	14.05±0.05	86.45±9.00	15.82±1.73	69.64±0.43[b]	50.33
Ischemia 45 min	14.90±5.83	91.17±6.32	13.08±5.29	58.80±6.88[b]	58.06
Ischemia 60 min	18.97±10.15	87.74±6.77	17.27±0.43	48.34±0.43[b]	65.52

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM of three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control value $p < 0.05$ by group t-test.

[c]Percent decrease from Control values.

Table 15:EFFECT OF 15-60 min ISCHEMIA ON g-GLUTAMYL TRANSFERASE ACTIVITY IN CH AND BBMV(s) ISOLATED FROM JUXTAMEDULLARY CORTEX.

	CH	BBM			
CONTROL	33.14 ± 2.67[a]		320.21 ± 20.00		
	CONTRALATERAL		ISCHEMIA		
	CH	BBM	CH	BBM	% decrease[c]
Ischemia 15 min	37.78±12.81	312.49±28.23	37.70±9.42	221.67±5.50[b]	30.77
Ischemia 30 min	31.32±3.60	291.02±13.90	35.88±1.07	151.01±2.54[b]	52.84
Ischemia 45 min	35.36±14.87	241.06±20.62	33.03±9.40	116.11±14.08[b]	63.71
Ischemia 60 min	38.98±17.33	254.13±8.93	31.43±12.85	104.91±21.04[b]	67.24

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM of three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control value $p < 0.05$ by group t-test.

[c]Percent decrease from Control values.

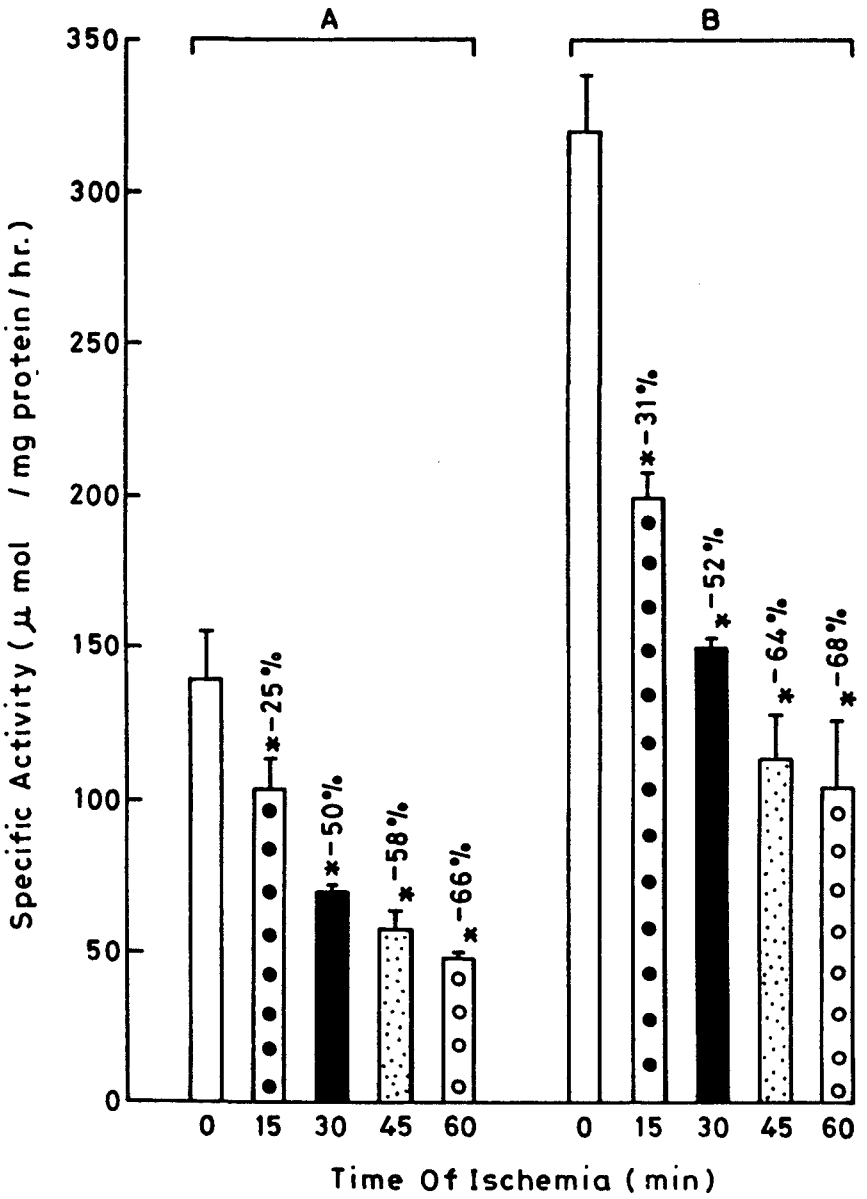


Fig.7: Specific Activity of GGTase in BBMVs from (A) Superficial cortex (BBMV-SC) and (B) Juxtamedullary cortex (BBMV-JMC) after 15-16 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p < 0.05$ or higher degree by group t-test.

Table 16: EFFECT OF 30 min ISCHEMIA ON KINETIC PARAMETERS OF ALKALINE PHOSPHATASE IN BBMV-SC AND BBMV-JMC.

	BBMV-SC			BBMV-JMC		
	Km x 10^{-4} M	Vmax umoles/mgpr/hr	% change[b] (Vmax)	Km x 10^{-4} M	Vmax umoles/mgpr/hr	% change[b] (Vmax)
Control	3.18	75.76		4.07	71.43	
Ischemia 30 min	2.26	45.25	-40%	1.88	38.31	-46%

[a]Values are Mean for three different preparations.

[b]Percent change from normal values.

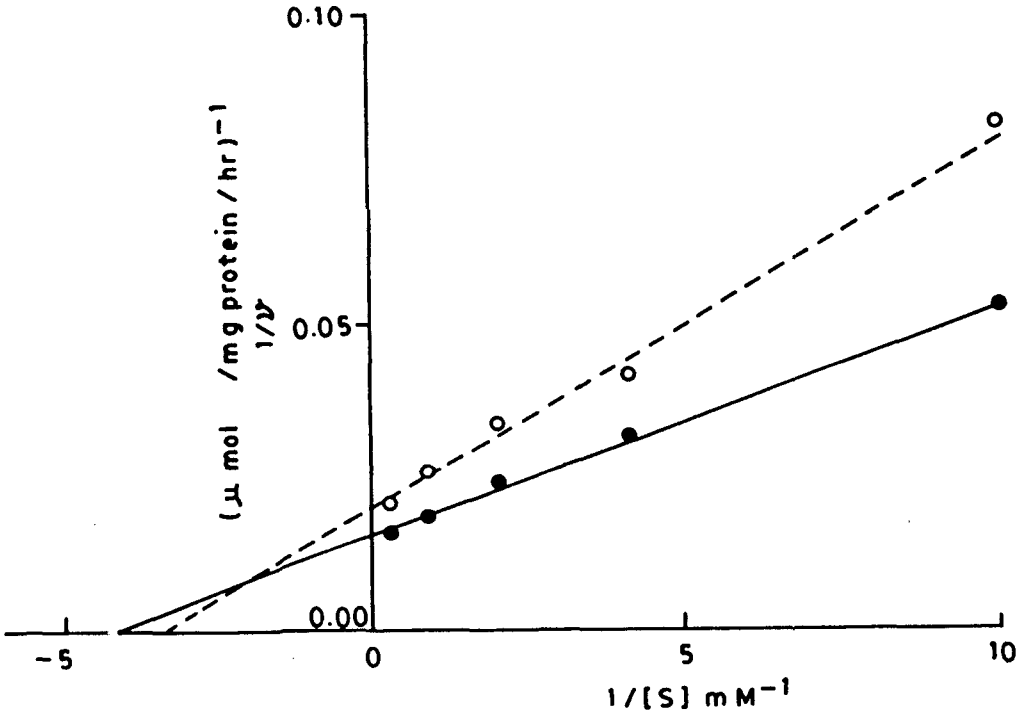


Fig.8: Kinetics of AlkPase in BBMV-SC after 30 min ischemia evaluated by Lineweaver-Burk plot. Normal (—●—) , ischemia (--o--).

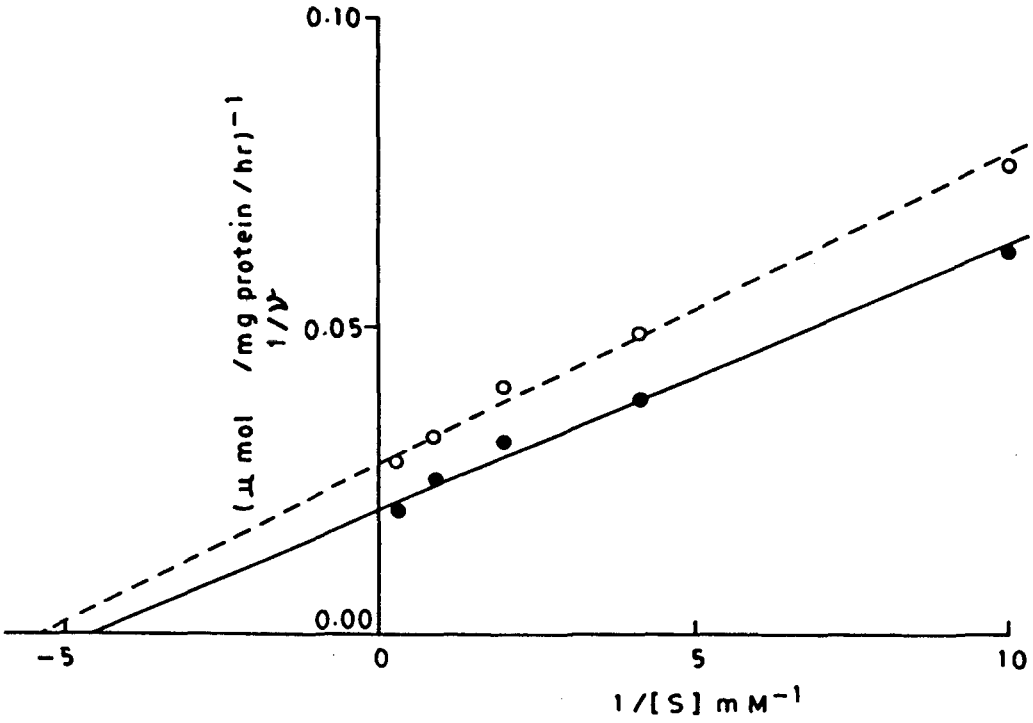


Fig.9: Kinetics of AlkPase in BBMV-JMC after 30 min ischemia evaluated by Lineweaver-Burk plot. Normal (—●—), ischemia (--o--).

Table 17:EFFECT OF 30 min ISCHEMIA ON KINETIC PARAMETERS OF g-GLUTAMYL TRANSFERASE IN BBMV-SC AND BBMV-JMC.

	BBMV-SC			BBMV-JMC		
	Km x 10^{-4} M	Vmax umol/mgpr/hr	% change[b] (Vmax)	Km x 10^{-4} M	Vmax umol/mgpr/hr	% change[b] (Vmax)
Normal	2.28	312.50		2.36	606.06	
Ischemia 30 min	1.78	111.11	-64%	2.22	352.11	-42%

[a]Values are Mean for three different preparations.

[b]Percent change from Control values.

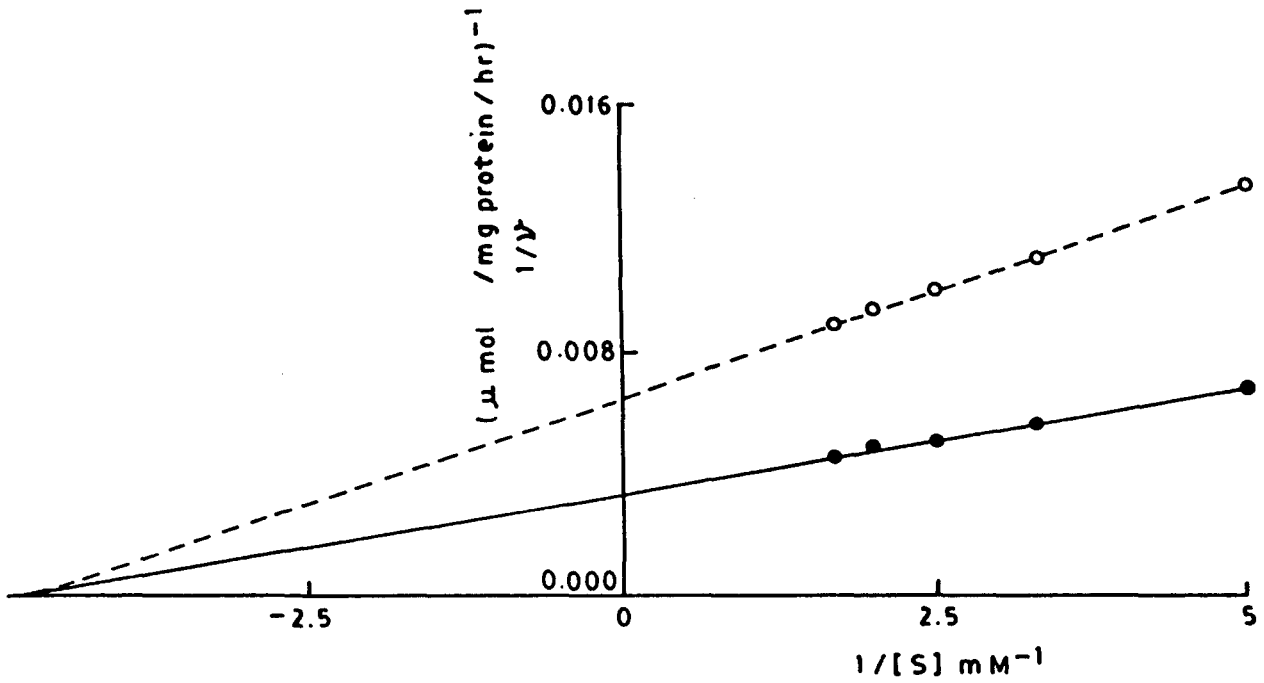


Fig.10: Kinetics of GGTase in BBMV-SC after 30 min ischemia, evaluated by Lineweaver-Burk plot. Normal (—●—), ischemia (--o--).

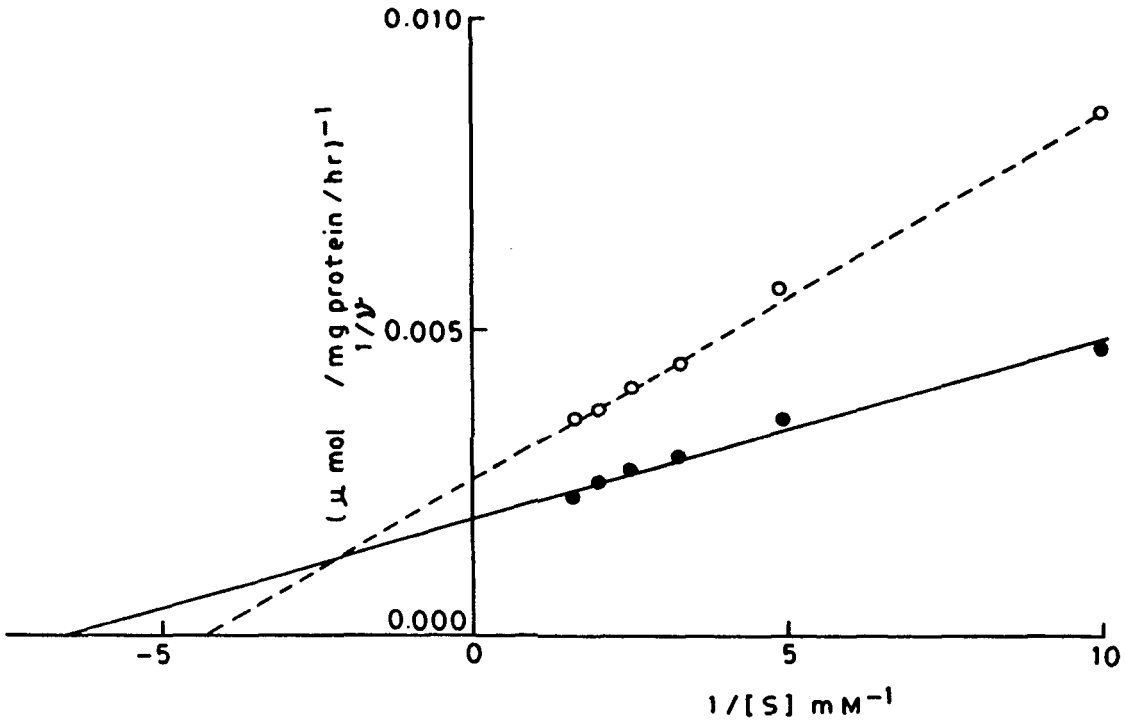


Fig.11: Kinetics of GGTase in BBMV-JMC after 30 min ischemia, evaluated by Lineweaver-Burk plot Normal (—●—), ischemia (--o--).

Table 18 : EFFECT OF 15 min ISCHEMIA and REFLOW OF DIFFERENT TIME PERIODS ON ALKALINE PHOSPHATASE ACTIVITY IN BBMV(s) ISOLATED FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY (JMC) CORTEX.

TIME OF REFLOW	BBMV-SC		BBMV-JMC	
	ISCH/REFLOW	CONTRALATERAL	ISCH/REFLOW	CONTRALATERAL
02 min	66.96±1.69[a]	81.51±2.08	72.09±4.52	84.27±2.03
15 min	55.95±0.84[b]	87.80±9.61	56.92±2.33[b]	91.28±3.29
60 min	69.78±0.88[c]	87.02±3.16	63.37±3.43[c]	87.21±2.15
120 min	75.95±4.31[c]	88.70±3.95	73.90±3.24[c]	90.89±3.60

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM of data from three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

Values for BBMV-SC and BBMV-JMC from controls were 100.37±2.19 and 109.40±9.81 respectively.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

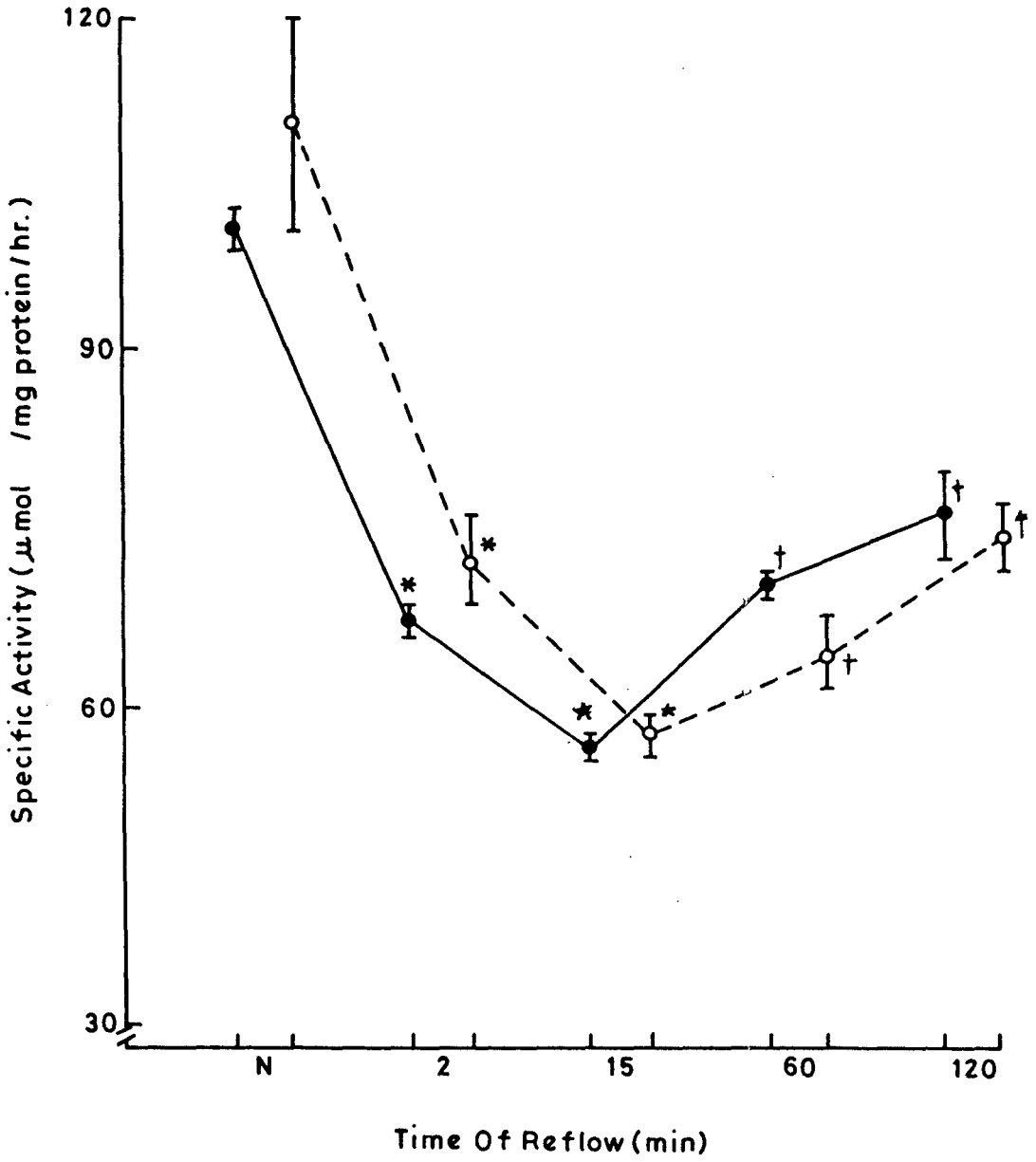


Fig.12: Specific activity of AlkPase in BBMV-SC (—●—) and BBMV-JMC (--o--) after 15 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

Table 19: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON ALKALINE PHOSPHATASE ACTIVITY IN BBMV(s) FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY CORTEX (JMC).

TIME OF REFLOW	BBMV-SC		BBMV-JMC	
	ISCH/REFLOW	CONTRALATERAL	ISCH/REFLOW	CONTRALATERAL
02 min	55.67±4.69[a]	102.40±4.89	64.65±1.63	100.72±3.88
15 min	43.56±3.59[b]	90.98±2.67	42.02±1.84[b]	89.89±4.09
60 min	63.15±0.86[c]	81.97±0.05	52.73±2.74[c]	86.96±0.50
120 min	72.75±2.87[c]	74.78±1.00	58.02±1.00[c]	87.83±1.23

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM of data from three different preparations. Each preparation kidneys from includes 5-6 rats in each group.

Values for BBMV-SC and BBMV-JMC from controls were 100.37±2.19 and 109.40±9.81 respectively.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

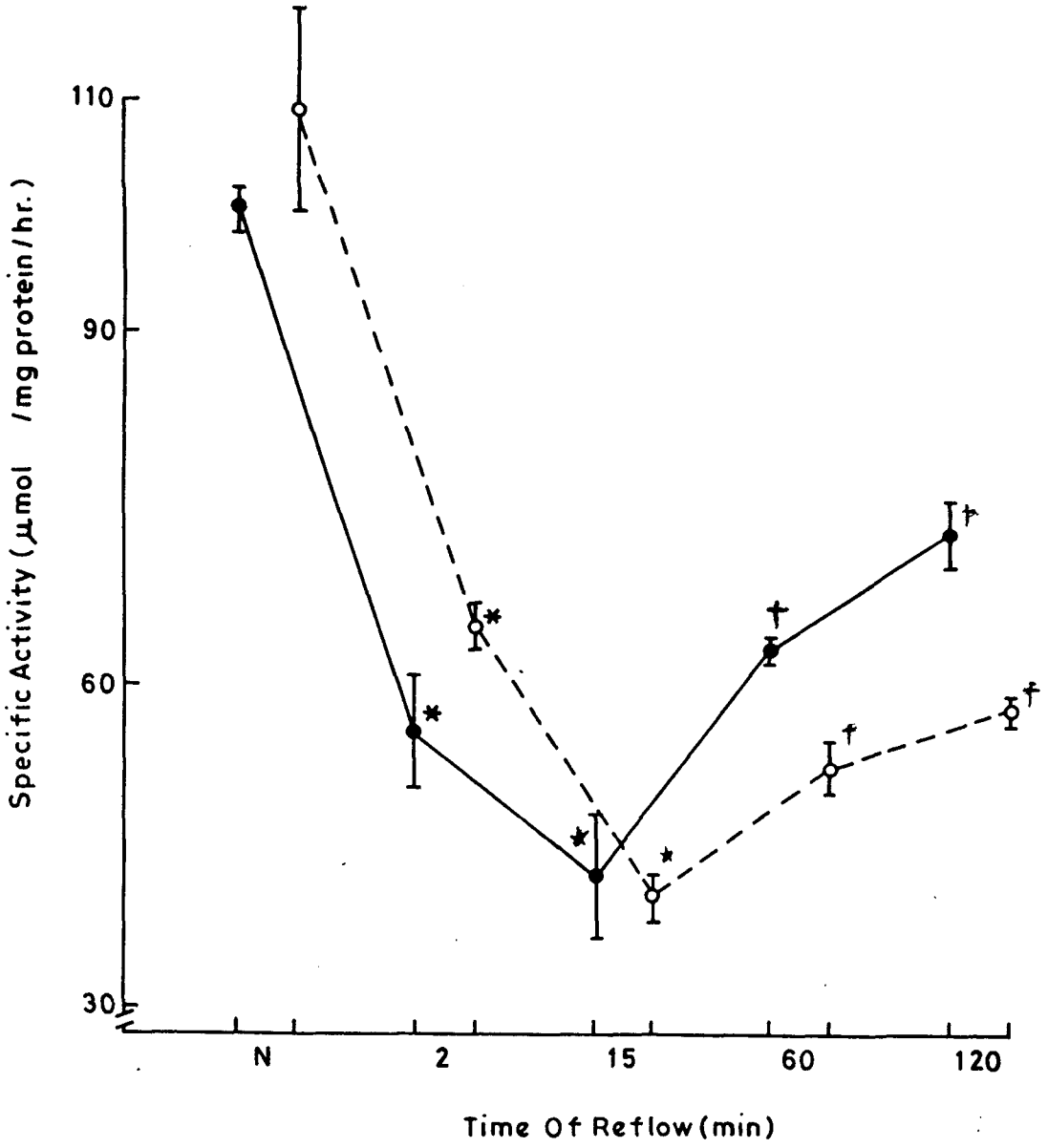


Fig.13: Specific Activity of AlkPase in BBMV-SC (—●—) and BBMV-JMC (--o--) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

Table 20:EFFECT OF 15 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON g-GLUTAMYL TRANSFERASE ACTIVITY IN BBMV(s) ISOLATED FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY CORTEX (JMC).

TIME OF REFLOW	BBMV-SC		BBMV-JMC	
	ISCH/REFLOW	CONTRALATERAL	ISCH/REFLOW	CONTRALATERAL
02 min	67.80±1.15[a]	86.39±3.85	152,93±5.71	242.97±8.14
15 min	53.23±1.44[b]	93.89±5.62	122.49±4.58[b]	329.39±7.55
60 min	65.10±1.90[c]	94.50±2.29	173.14±4.58[c]	268.82±4.16
120 min	65.41±2.11[c]	95.17±2.73	174.63±4.59[c]	292.96±3.46

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM for three different preparations.Each preparation includes kidneys from 5-6 rats in each group.

Values for BBMV-SC and BBMV-JMC from controls were 140.21±17.44 and 320.21±20.00 respectively.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

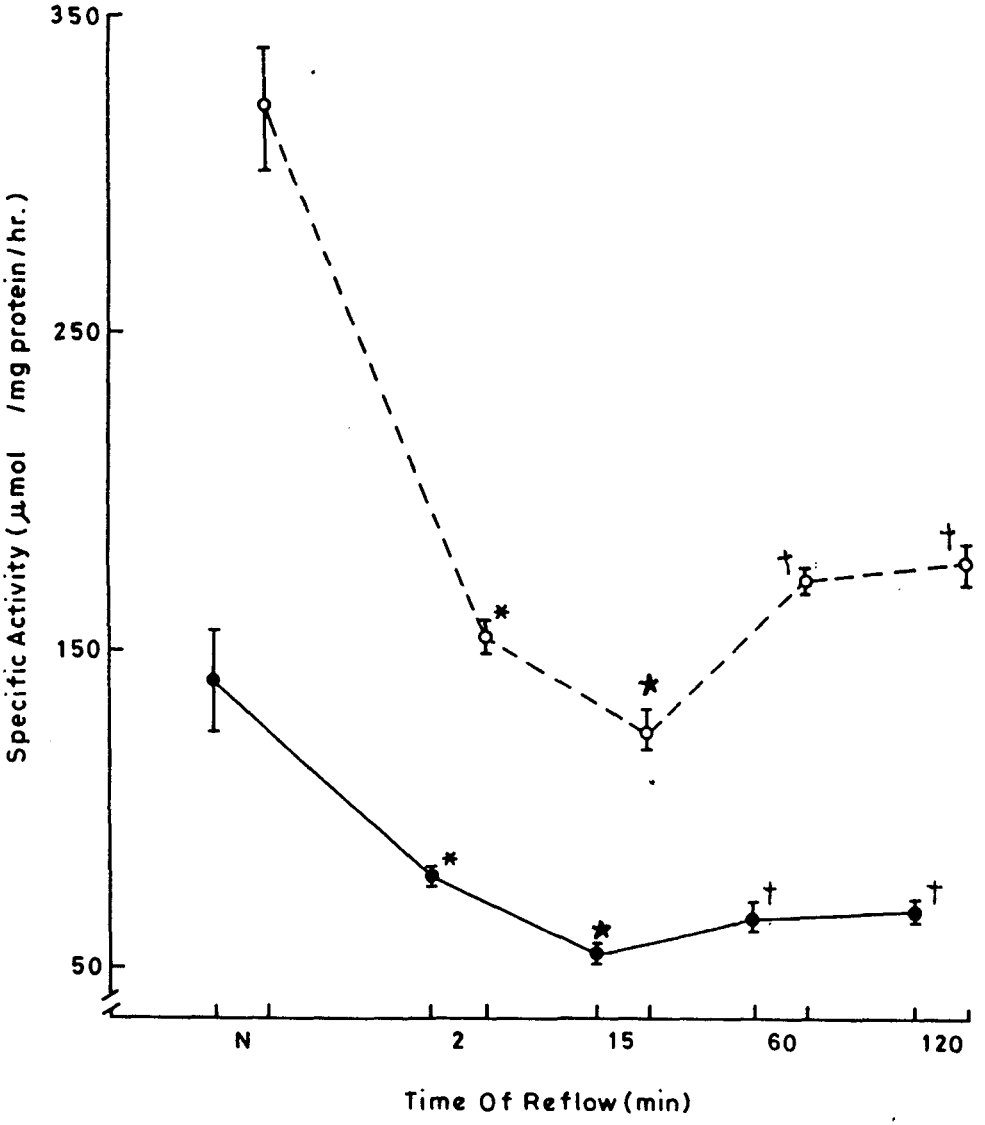


Fig.14: Specific Activity of GGTase in BBMV-SC (—●—) and BBMV-JMC (--o--) after 15 min ischemia and refow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

Table 21: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON g-GLUTAMYL TRANSFERASE ACTIVITY IN BBMV(s) FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY CORTEX (JMC).

TIME OF REFLOW	BBMV-SC		BBMV-JMC	
	ISCH/REFLOW	CONTRALATERAL	ISCH/REFLOW	CONTRALATERAL
02 min	45.35±1.43[a]	123.00±7.36	153.71±12.88	254.19±6.78
15 min	40.72±1.63[b]	94.52±1.94	114.31±5.42[b]	309.13±12.92
60 min	50.44±1.24[c]	100.00±2.05	132.31±5.76[c]	351.60±9.04
120 min	50.15±1.73[c]	98.55±2.05	142.18±4.89[c]	327.05±7.70

[a]Activities(umol/mg protein/hr) are expressed as Mean±SEM for three different preparations.Each preparation includes kidneys from 5-6 rats in each group.

Values for BBMV-SC and BBMV-JMC from controls were 140.21±17.44 and 320.21±20.00 respectively.

[b]Significantly different from 2 min Reflow group $p<0.05$ by group t-test.

[c]Significantly different from 15 min Reflow group $p<0.05$ by group t-test.

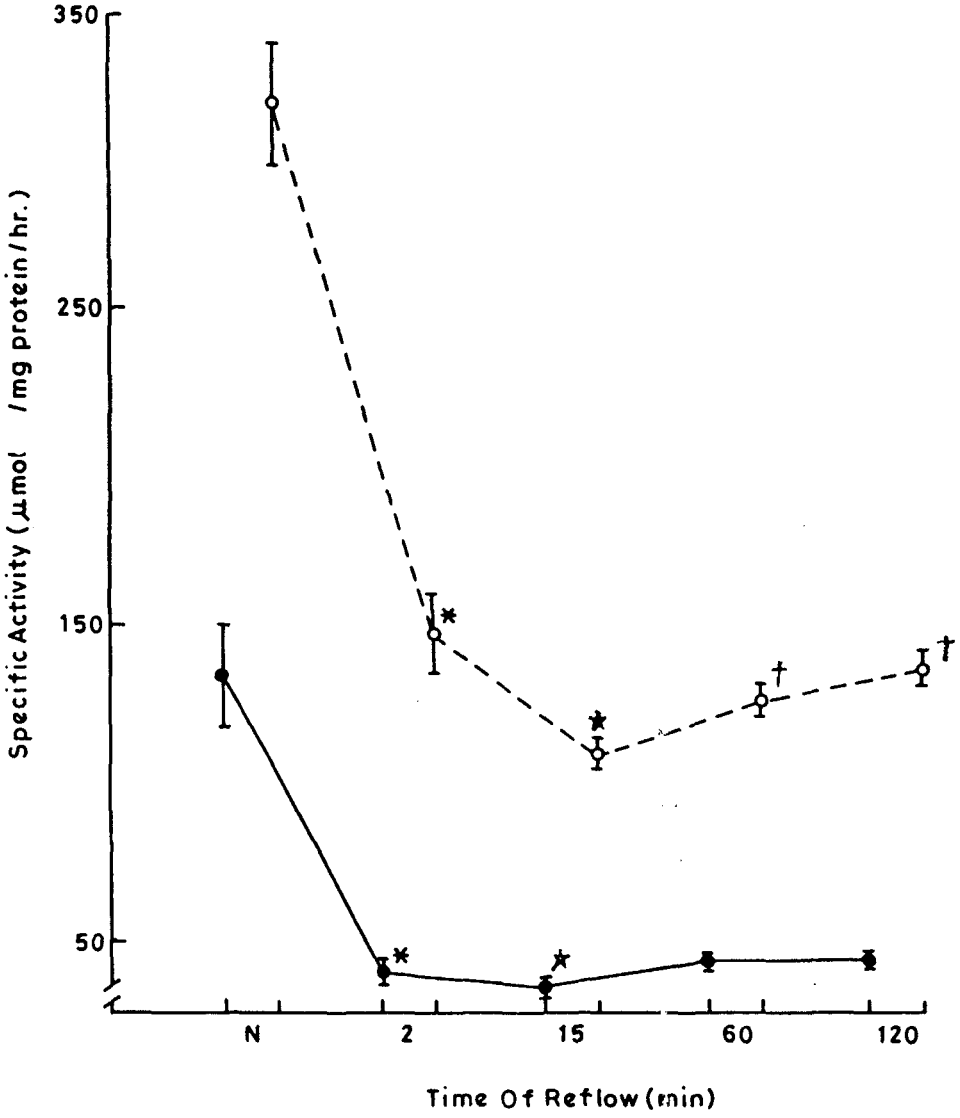


Fig.15: Specific Activity of GGTase in BBMV-SC (—●—) and BBMV-JMC (--o--) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

When the activities of both AlkPase (Fig. 16) and GGTase (Fig. 17) were further analysed, the total activities of the membrane-bound enzymes declined while in the supernatant (free or released enzymes) were found increased with the increase in the duration of ischemia in both SC and JM cortex. However, upon reflow of blood upto 120 min the activities of membrane bound enzymes increased in a time dependent manner and the free or released enzymes initially decreased rapidly after 30 min of ischemia, and further decreased to a lesser extent after 60 min of ischemia. The decline in membrane bound enzymes and increase in free enzymes were greater in JM-cortex as compared to SC-cortex. These result indicate that damage to the nephronal membranes occurred differentially in the membrane of different enzymes by ischemia while they were partially repaired and/or resynthesized after reflow also differentially (Table 22 & 23).

BBMV(s) purity was checked by analysing the activities of Na^+-K^+ ATPase (BLM enzyme) and acid phosphatase (lysosomal enzyme), the results (Table 24 & 25) indicate that the membrane preparation was several fold purified as the activities of Na^+-K^+ ATPase and acid phosphatase were not enriched similar to those of BBM marker enzymes.

TABLE-22: EFFECT OF ISCHEMIA (30 and 60 min) AND REFLOW OF 15 and 120 min ON TOTAL ACTIVITY- BOUND AND FREE AlkPase IN THE HOMOGENATES FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY (JMC) CORTEX.

Time of Reflow	CONTROL							
	SC		JM					
	Bound	Free	Bound	Free				
	841.98±19.60[A]	125.95±2.95	954.00±19.77	120.25±6.68				
	30 min Isch.				60 min Isch.			
	SC		JM		SC		JM	
	Bound	Free	Bound	Free	Bound	Free	Bound	Free
15 min	494.19±17.70[b]	234.52±2.82[b]	491.52±6.17[b]	322.38±6.90[b]	220.34±2.18[b]	273.00±18.81[b]	214.34±7.08[b]	460.99±5.12[b]
120 min	586.05±22.83[c]	165.13±6.94[c]	579.00±6.93[c]	243.21±9.63[c]	279.97±16.87[c]	333.00±9.74[c]	235.33±6.72[b]	435.85±13.35

[a] Activities are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b] Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c] Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

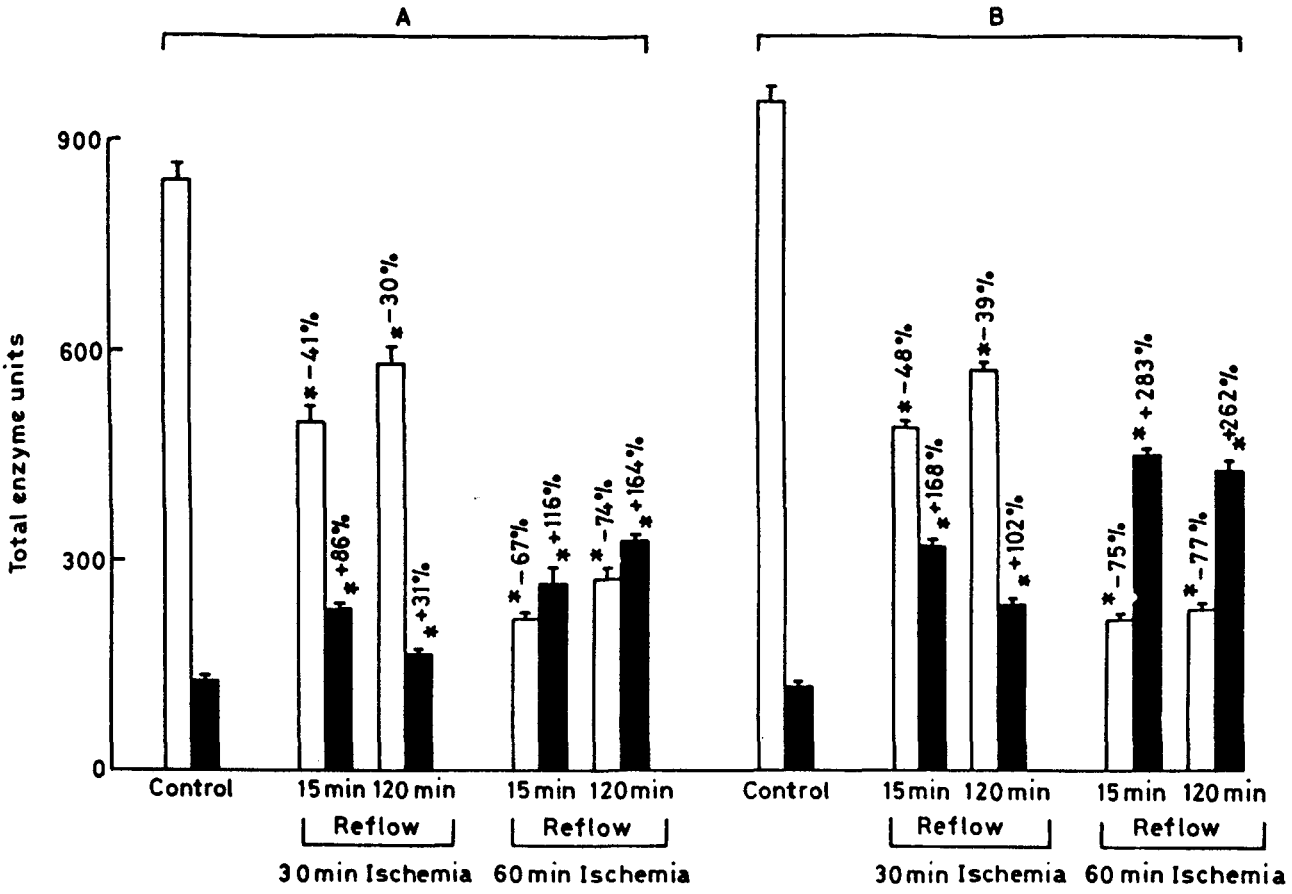


Fig.16: Total enzyme activity of AlkPase in (A) SC-cortex and (B) JMC-cortex, Bound (□), Free (■) after 30 and 60 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

TABLE-23: EFFECT OF ISCHEMIA (30 and 60 min) AND REFLOW OF 15 and 120 min ON TOTAL ACTIVITY- BOUND AND FREE GGTase IN HOMOGENATES FROM SUPERFICIAL (SC) AND JUXTAMEDULLARY (JMC) CORTEX.

Time of Reflow	CONTROL			
	SC		JM	
	Bound	Free	Bound	Free
	1722.61±53.61[a]	363.30±8.82	4510.24±83.50	714.28±22.49

	30 min Isch.				60 min Isch.			
	SC		JM		SC		JM	
	Bound	Free	Bound	Free	Bound	Free	Bound	Free
15 min	751.65±28.26[b]	598.03±5.65[b]	1672.93±46.89[b]	1606.14±53.37[b]	482.58±4.87[b]	810.95±22.57[b]	1104.50±8.34[b]	2070.35±42.21[b]
120 min	1097.09±30.28[c]	439.00±18.02[c]	2832.23±19.61[c]	1006.83±35.63[c]	544.19±10.16[c]	893.40±18.25[c]	1084.52±7.98[b]	2026.84±49.90[b]

[a]Activities are expressed as Mean±SEM for 3-6 different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

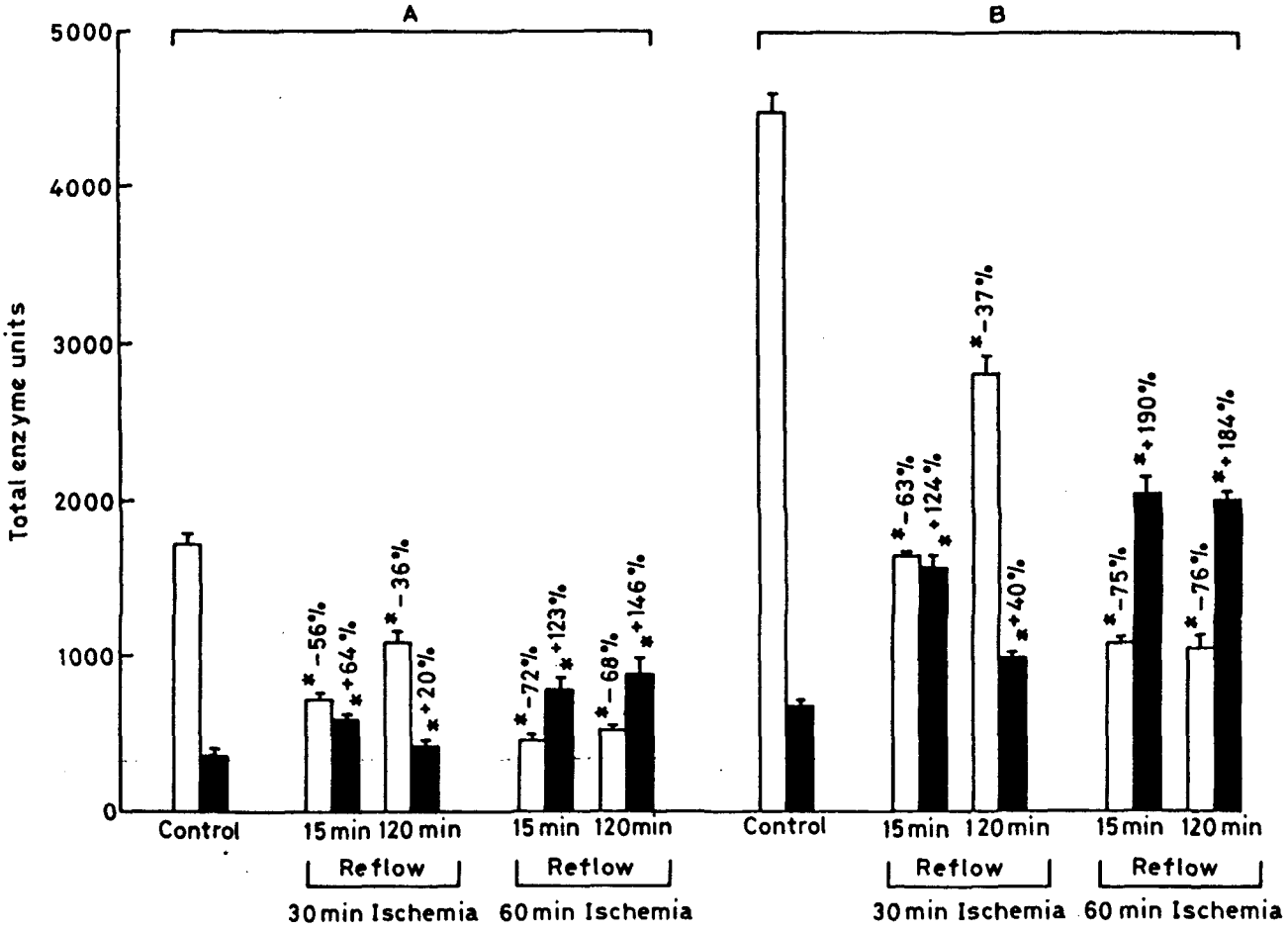


Fig.17: Total enzyme activity of GGTase in (A) SC-cortex and (B) JMC-cortex, Bound (□), Free (■) after 30 and 60 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

Table 24: ACTIVITY OF Na+/K+ ATPase IN BBM AS RATIO OF CH

	SC		JMC	
CONTROL	0.55		0.36	
	ISCHEMIA	CONTRALATERAL	ISCHEMIA	CONTRALATERAL
Ischemia 15 min	0.43	0.27	0.38	0.21
Ischemia 30 min	0.31	0.44	0.30	0.33
Ischemia 45 min	0.20	0.24	0.13	0.28
Ischemia 60 min	0.29	0.65	0.61	0.47

Activity is represented as the ratio of activity in BBM to the activity in CH.

Table 25: ACTIVITY OF ACID PHOSPHATASE IN BBM AS RATIO OF CH.

	SC		JMC	
CONTROL	0.27		0.30	
	ISCHEMIA	CONTRALATERAL	ISCHEMIA	CONTRALATERAL
Ischemia 15 min	0.39	0.33	0.30	0.59
Ischemia 30 min	0.29	0.25	0.25	0.44
Ischemia 45 min	0.35	0.20	0.31	0.20
Ischemia 60 min	0.43	0.48	0.43	0.45

Activity is represented as the ratio of activity in BBM to the activity in CH.

DISCUSSION - I

It is well established that loss of renal epithelial cell viability due to ischemic ARF resulted in the accumulation of cellular debris from injured tubule cells (85,93) which leads to cell necrosis and death and renal excretory functions are altered (85). The proximal tubular segment of the nephron was shown to be the chief site of the damage that occurred due to ischemic or toxic insult (7,130,203,204). Histological evidence shows that the damage due to ARF primarily occurs in the pars recta (the S_3 segment) in animal models of ischemic injury (7,85). The kidney responds to ischemia with effacement of the proximal tubular brush border membrane as evident by the marker enzymes activities associated with this membrane (7,8).

The primary aim of the present study was to localize the effect of ischemia on the proximal tubular brush border membrane (BBM), the major functional site, isolated from superficial (SC) and deep (JMC) rat renal cortex, after different duration of ischemia and blood reflow. The activities of BBM marker enzymes: alkaline phosphatase (AlkPase) and g-Glutamyl transferase (GGTase) were determined to examine the structural and functional integrity of proximal tubules under ischemic and reflow conditions. The serum concentrations of Cr, Pi, PL, and Chol

(Table 1)were increased progressively with different durations of ischemia and brought back towards normal values in certain situations of blood reflow (Table 2 , 3). The activities of AlkPase (Table 4; Fig. 3A) and GGTase (Table 5; Fig. 3B) in the BBMV(s) isolated from whole cortex declined markedly with 15-60 min ischemia and were in agreement with the earlier studies (8,9). Further analyses showed that the activities of AlkPase and GGTase were increased in the supernatent with a corresponding decrease in the pellet of cortical homogenate (membrane-bound enzyme) (Table 10 & 11; Fig. 5) indicates that BBM is severly damaged and may have been partially effaced during ischemia and the enzymes and other components might have dissociated from the BBM and released in the supernatent. This might also explain the increase in the activity of GGTase in urine (bound & free) observed by Herminghuysen et al. (160) and Desmouliere and Cambar (161). The activity pattern in BBMV(s) isolated from SC and JMC indicates that the activities of these marker enzymes decreased due to ischemia to a greater extent in BBMV-JMC than in BBMV-SC (Table 12-15; Fig. 6 & 7). As observed in the whole cortical homogenates the activities of AlkPase and GGTase were also

increased in supernatent or free enzymes and declined in the membrane bound fraction of SC and JM cortex (Table 22 & 23). The decrease in membrane bound enzyme and the corresponding increase in the free enzyme was greater in JM than in SC cortex (Fig. 16 & 17). The data is in general agreement with the earlier morphological and some biochemical studies (7-9,132,203) showing greater ischemic damage to the pars recta (S_3 subsegment) segment of the proximal tubule. Further, the decline in activities of both AlkPase and GGTase were largely due to decrease in Vmax with no or small effect of Km values (Table 16 & 17; Fig. 8-11) which indicate that the decrease may be due to loss of active enzyme molecules bound per unit of BBM isolated from ischemic kidneys compared to control preparations. It has been demonstrated (7,8,205) that damage caused to the brush border membranes of the kidney due to ischemia is reversible and is associated with reversible decrease in the membrane associated enzyme specific activities and morphological changes in the proximal tubule segment upon reflow of blood (7,9). The reversibility of ischemic acute renal failure depends on renal epithelial cell regeneration to reconstruct normal nephronal architecture so as to reestablish normal functioning of the kidney (18). It has also been reported

that both the damage to the cell as well as regeneration of tubular cells depends on the duration of ischemia and blood reflow (18). The findings of the present study demonstrate the reversal of the serum concentrations of Cr and Pi after 120 min reflow after 15 and 30 min ischemic injury. However, the reversal was incomplete (Table 2 & 3). A significant recovery of the specific activities of both AlkPase and GGTase was observed after 120 min of reflow in 15 and 30 min ischemic BBMV(s) isolated from whole cortex (Table 6 & 8; Fig. 4), and SC and JM cortex (Table 18-21; Fig. 12-15) compared to values of ischemic rats. However, there was very small recovery in the specific activities of both the marker enzymes following 60 min of ischemia followed by blood reflow (Table 7 & 9; Fig. 4). These results indicates that 15-30 min ischemia causes to some extent reversible damage while 60 min ischemia causes greater damage which was largely irreversible.

The results also indicate that early reflow of 15 min after all ischemic periods showed the maximum decrease in enzyme activities. The activities of both AlkPase and GGTase in BBMV(s) from whole cortex, SC and JMC declined further after 15 min reflow as compared to ischemic rats (2 min

reflow group) as was reported earlier (8,9). The recovery of AlkPase appeared to be different from that of GGTase both in BBMV-SC and BBMV-JMC while the recovery of AlkPase was relatively greater in BBMV-SC compared to BBMV-JMC (Fig. 12-15) after all ischemic periods studied. The recovery of GGTase appears to be more in BBMV-JMC as compared to BBMV-SC atleast in 15 min ischemic rats (Fig. 14 & 15). This might be due to the differential localization of AlkPase and GGTase in the BBM and differential susceptibility to ischemic injury (7,164,175). AlkPase, while located deeply on the cytoplasmic site of the membrane, GGTase is located in the middle of the BBM (162,164). Moreover, GGTase together with leucine amino-peptidase (LAP) is considered to be the marker enzyme of pars recta (S_3 subsegment) of the proximal tubules especially the juxtamedullary (JM) cortical region (162). Thus it can be considered that BBMV(s) of deep proximal tubular regions are greatly damaged due to ischemia and under reversible ischemic conditions regenerates at a slow rate than BBMV-SC. The present observations with regard to AlkPase and GGTase suggest that they are differentially affected both by ischemia and blood reflow.

The disappearance of brush border membrane is a

prominent index of renal damage. Various nephrotoxins such as cephaloridine (206), mercuric chloride (207), puromycin aminonucleoside (208), vinblastine (209) and uranyl nitrate (5) results in loss of microvilli from the proximal tubular cells and the damage caused by drugs were greater and regeneration processes required much longer time. Our studies indicate that if the damage is not severe enough to cause cellular necrosis, the BBM can be reformed and the integrity of the proximal tubules can be maintained by subsequent blood reflow of short duration after ischemic injury which is difficult to achieve in drug induced ischemic injury.

RESULTS & DISCUSSION
PART II

RESULTS - II

Effect of Ischemia on the activities of certain enzymes of carbohydrate metabolism

The function of the kidney i.e. the reabsorption of various ions and solutes depends on the continued sufficient energy supply as ATP. The generation of ATP is the contribution of several metabolic pathways including glycolysis and oxidative metabolism. The relationship between the O_2-tension and the major work function of the kidney as transport of Na^+ is well documented. The acute renal failure produced by ischemic insult resulted in the reduction of the O_2-tension in the kidney tissues due to the sluggish blood supply which is apparent from the variations of different enzymes of various metabolic pathways especially of glucose metabolism.

To determine the effect of ischemia on the enzymes of various metabolic pathways, the ischemia was produced by occluding the renal artery for various time periods e.g. 5, 15, 30, 60 min. After a brief reflow of 2 min, the kidneys were harvested and the activities of several enzymes e.g. LDH (glycolysis), MDH (TCA cycle), G6Pase and FDPase (gluconeogenesis) and G6PDH and ME (HMP-shunt pathway and the enzymes of biosynthetic importance) were determined in

the homogenates of the cortex and the medulla. The results are summarized in Table 26-31 (Fig. 18-23). A brief ischemia of 5 min caused an increase of LDH activity to similar extent both in the cortex (+15%) and the medulla (+19%). However, prolonged ischemia showed a decrease in LDH activity also to a similar extent both in the cortex and medulla and the decrease was linearly proportional to the time of ischemia. The maximum decline ($\geq$ 25%) was obtained after 60 min of ischemia (Table 26; Fig. 18A & B). The effect of ischemia on the MDH activity however was different from that of the LDH activity. At all time points of ischemia, the activity of MDH was lowered as a function of duration of ischemia. The effect was maximum after 60 min ischemia both in the cortex as well as the medulla (Table 27). However, the decrease in the activity of MDH appeared to be greater in the cortex compared to the medulla (Fig. 19 A & B).

The effect of ischemia was also determined on the activity of FBPase and G6Pase, the representative enzymes of gluconeogenesis. Similar to the effect on LDH activity the activity of FDPase was significantly increased after 5 min ischemia, however, to a greater extent in the cortex (+41%)

TABLE-26: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF LACTATE DEHYDROGENASE (LDH) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	1.290±0.014[a]	1.220±0.036	1.090±0.027	0.851±0.025
5 min	1.540±0.067[b]	1.410±0.030	0.981±0.020[b]	0.950±0.018[b]
15 min	1.090±0.025[b]	1.030±0.018[b]	0.796±0.040[b]	0.718±0.031[b]
30 min	1.050±0.014[b]	1.010±0.028[b]	0.682±0.014[b]	0.696±0.014[b]
60 min	0.977±0.057[b]	0.893±0.028[b]	0.650±0.045[b]	0.638±0.005[b]

[a]Activities (umol NADH oxidized/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

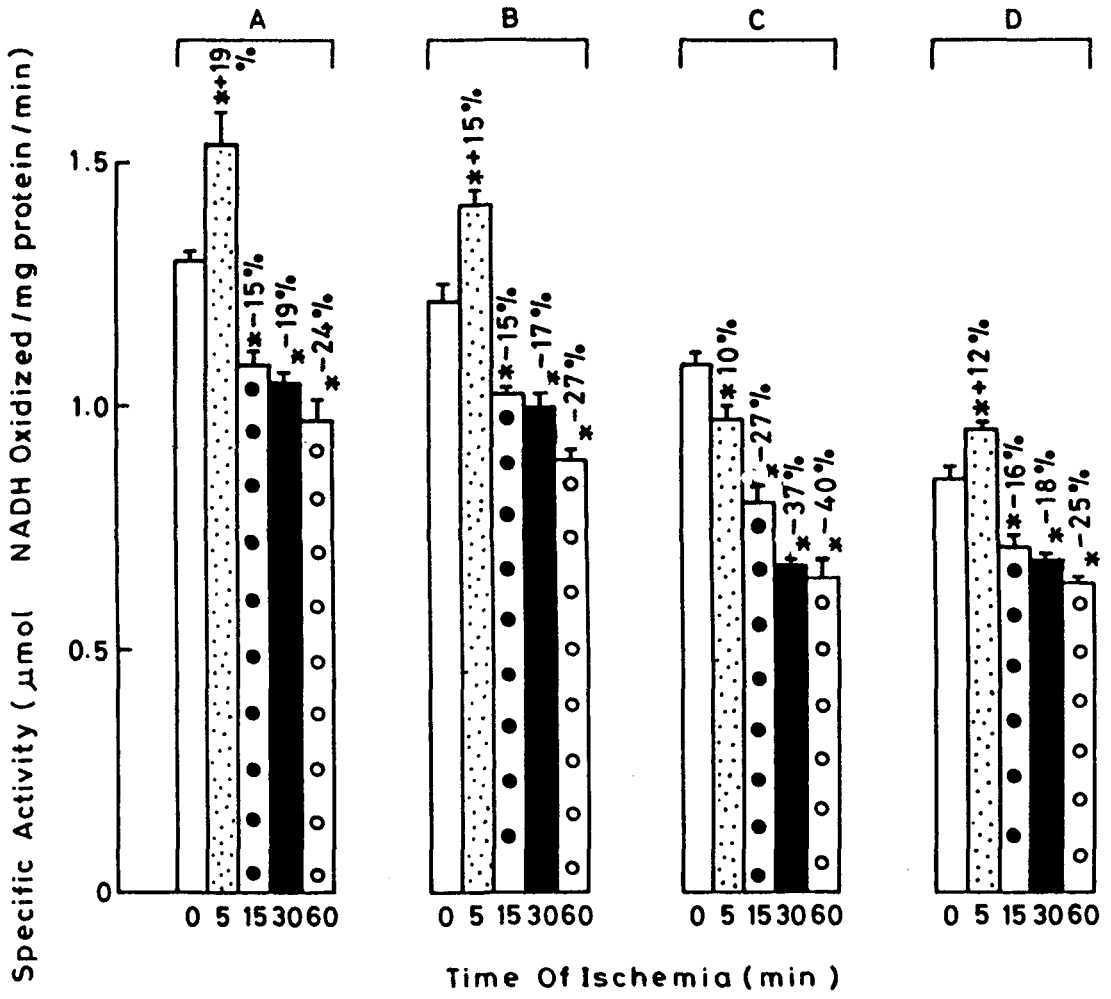


Fig.18: Specific Activity of LDH in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

TABLE-27: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF MALATE DEHYDROGENASE (MDH) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	5.72±0.011[a]	6.21±0.184	7.76±0.310	6.50±0.331
5 min	5.41±0.060[b]	6.06±0.092	7.04±0.079	6.49±0.201
15 min	5.00±0.028[b]	5.06±0.007[b]	4.64±0.044[b]	5.76±0.081
30 min	4.84±0.056[b]	4.62±0.007[b]	4.14±0.239[b]	4.30±0.081[b]
60 min	4.51±0.130[b]	4.56±0.007[b]	4.09±0.032[b]	4.24±0.108[b]

[a]Activities (umol NADH oxidized/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

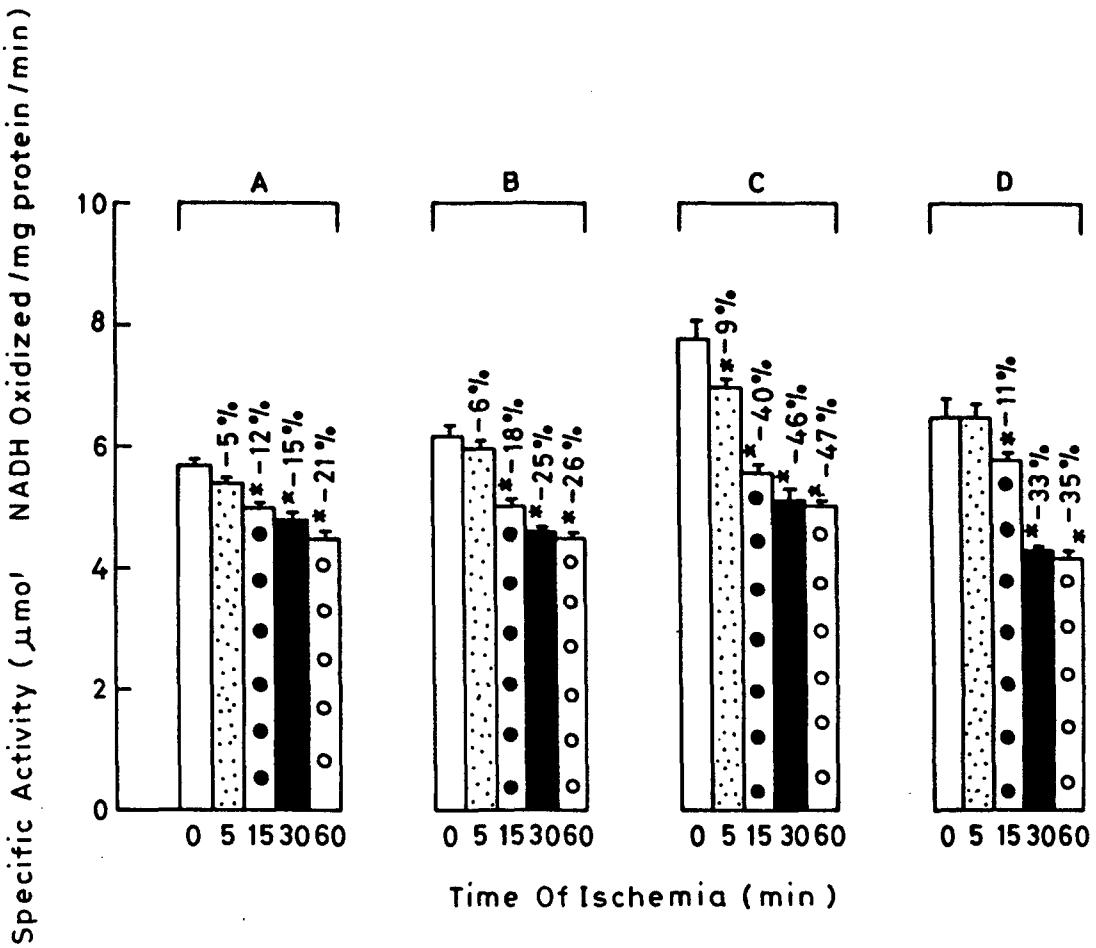


Fig.19: Specific Activity of MDH in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

than in the medulla (+17%) (Table 28). The effect of 15 min or 30 min ischemia did not produce any significant change in the cortex as compared to control non-ischemic rats. However, in the medulla the activity of FBPase was profoundly lowered both after 15 or 30 min of ischemia. After 60 min of ischemia the activity of FBPase declined moderately in the cortex (-18%) but in the medulla almost 50% of activity was lost (Table 28; Fig. 20 A & B). Similar effects of ischemia were observed on the activity of G6Pase (Table 29; Fig. 21 A & B) both in the cortex and medulla. A 5 min ischemia caused an enhancement of the activity again to a greater extent in the cortex (+31%) than in the medulla (+17%) while prolonged ischemia for 60 min caused significant decline in the activity of G6Pase and to a greater extent in the medulla (-22%) than the cortex (-12%) (Table 29; Fig. 21 A & B) similar to those of FBPase activity.

The effect of ischemia was also determined on the activities of G6PDH (an enzyme of HMP-shunt pathway) and ME (an enzyme of biosynthetic importance together with G6PDH, which supply NADPH). The results indicate that the activity of G6PDH was lowered with the increasing duration of

TABLE-28: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF FRUCTOSE-1,6-BISPHOSPHATASE (FBPase) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	0.197±0.002[a]	0.453±0.005	1.01±0.012	1.005±0.067
5 min	0.231±0.003[b]	0.639±0.005[b]	1.11±0.017[b]	1.07±0.003
15 min	0.131±0.002[b]	0.441±0.007[b]	0.915±0.018[b]	0.898±0.005[b]
30 min	0.127±0.001[b]	0.433±0.005[b]	0.863±0.005[b]	0.815±0.001[b]
60 min	0.104±0.002[b]	0.369±0.003[b]	0.784±0.003[b]	0.782±0.015[b]

[a]Activities (umol Pi released/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

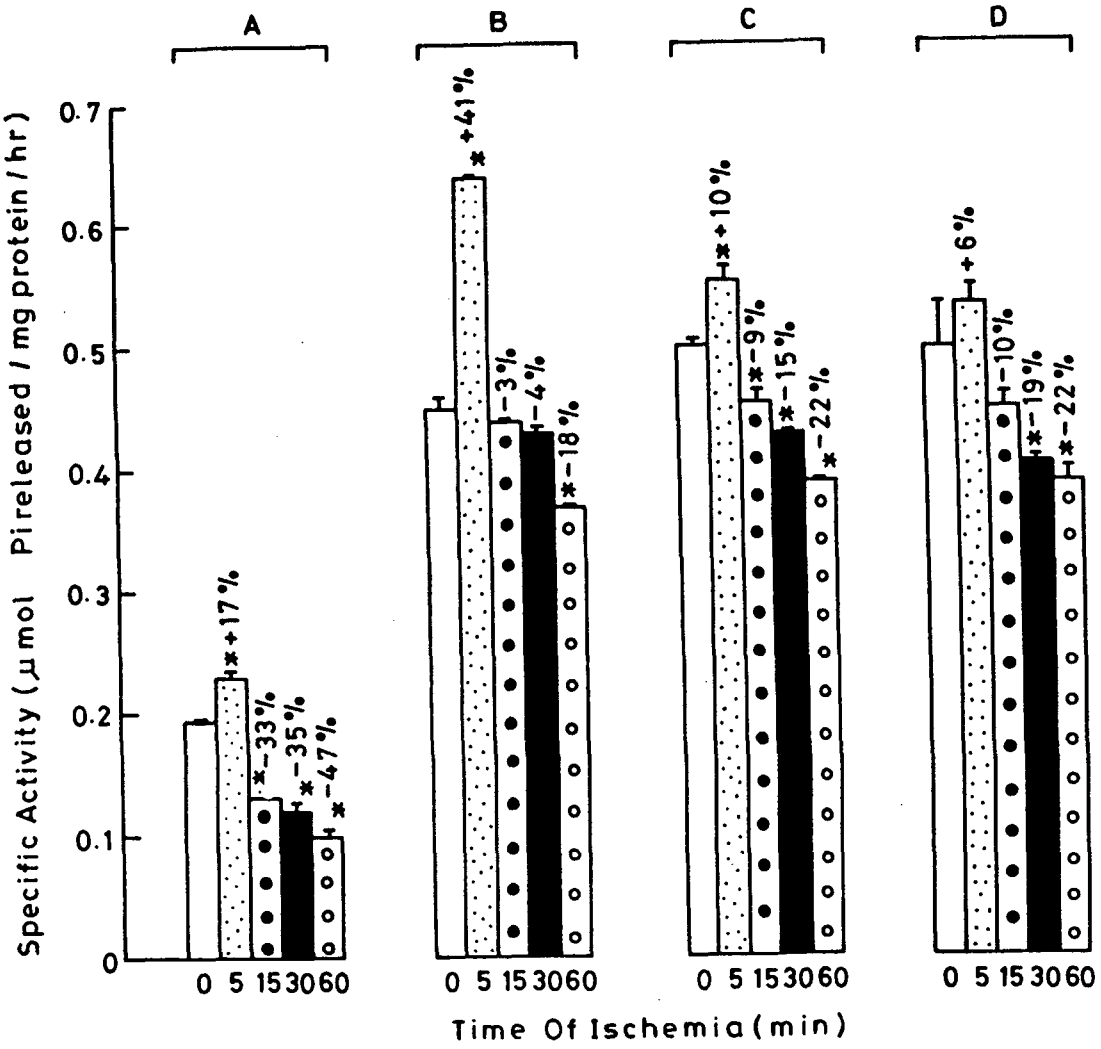


Fig.20: Specific Activity of FBPase in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min ischemia.

Values are represented as Mean ±SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

TABLE-29: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATASE (G6Pase) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, and JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	0.067±0.001[a]	0.116±0.001	0.321±0.008	0.281±0.009
5 min	0.075±0.001[b]	0.152±0.001[b]	0.338±0.003	0.330±0.008[b]
15 min	0.064±0.001[b]	0.112±0.001[b]	0.262±0.009[b]	0.248±0.022[b]
30 min	0.062±0.001[b]	0.103±0.001[b]	0.254±0.008[b]	0.240±0.008[b]
60 min	0.052±0.001[b]	0.102±0.002[b]	0.221±0.008[b]	0.239±0.001[b]

[a]Activities (umol Pi released/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

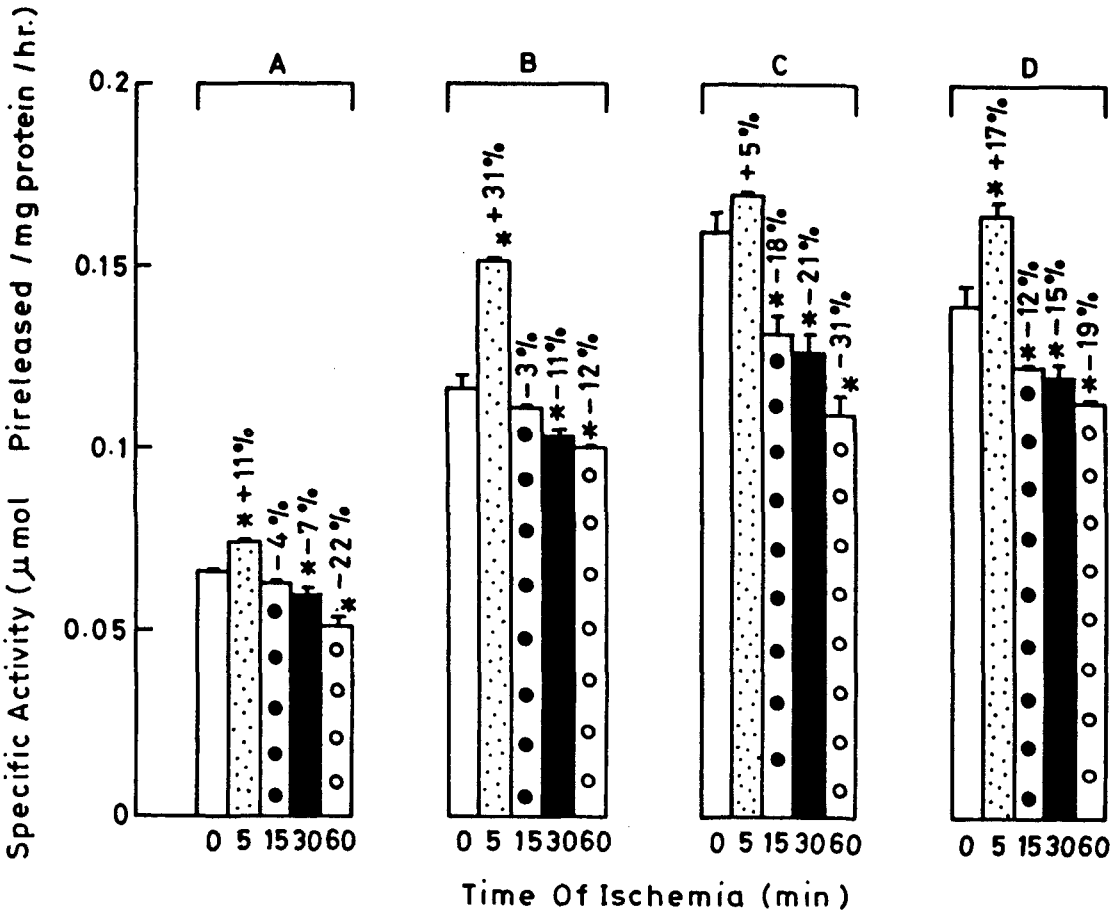


Fig.21: Specific Activity of G6Pase in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JM-cortex after 5-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

ischemia. The effect was significantly greater in medullary enzyme than the cortex ones (Table 30; Fig. 22 A & B). However, the activity of ME was slightly increased (+10%) in the cortex while it was decreased in the medulla (-17%) after 5 min ischemia. Prolonged ischemia (15-60 min) showed a significant decline in the activity of the enzyme and the effect was always greater in the medulla than in the cortex (Table 31; Fig. 23 A & B).

The activity of Na^+/K^+ ATPase, a BLM enzyme was similarly affected by the ischemia both in the cortex and the medulla and the effect was linearly proportional to the duration of the occlusion of the renal artery (Table 32; Fig. 24 A & B).

The activities of the above metabolic enzymes were also determined in the kidney tissues taken from the outer cortex (superficial cortex) and from the deep cortex plus outer stripe of outer medulla (JMC) as the BBMV(s) would be isolated from these tissues to study the effect of ischemia on transport functions. It is believed that the SC-tissue predominantly contains the early proximal convoluted tubule (S_1 and S_2-subsegments) together with DCT and CCT while the JMC-tissue contains predominantly the late proximal tubule

TABLE-30: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, and JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	0.381±0.001[a]	0.143±0.006	0.181±0.004	0.280±0.008
5 min	0.326±0.001[b]	0.142±0.001	0.296±0.014[b]	0.398±0.004[b]
15 min	0.259±0.016[b]	0.121±0.001[b]	0.146±0.011[b]	0.216±0.011[b]
30 min	0.213±0.022[b]	0.114±0.007[b]	0.125±0.001[b]	0.184±0.007[b]
60 min	0.205±0.004[b]	0.077±0.007[b]	0.112±0.004[b]	0.177±0.011[b]

[a]Activities (umol $NADP^+$ reduced/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

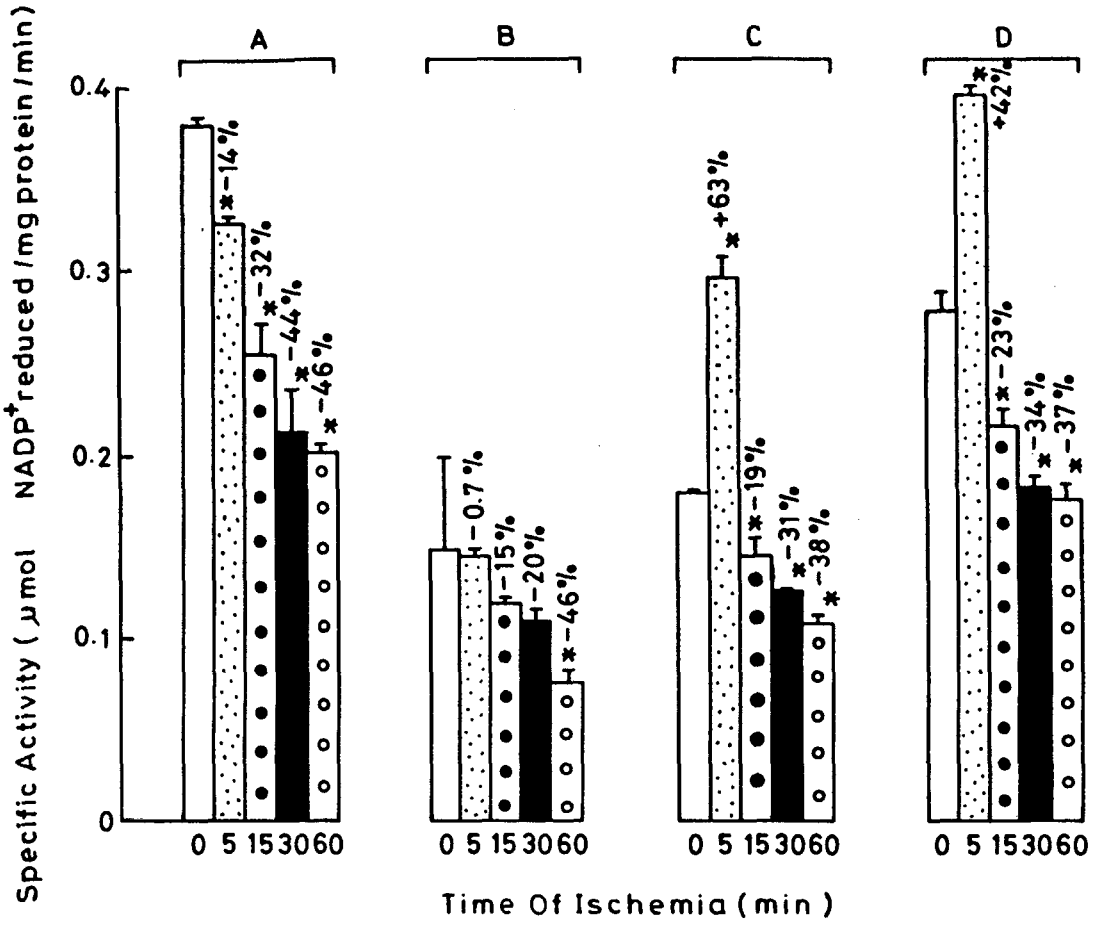


Fig.22: Specific Activity of G6PDH in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

TABLE-31: EFFECT OF DIFFERENT TIME PERIODS OF ISCHEMIA ON THE ACTIVITY OF MALIC ENZYME (ME) IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedull-ary Cortex
0 min	0.446±0.014[a]	0.123±0.002	0.280±0.01	0.404±0.028
5 min	0.369±0.013[b]	0.130±0.003	0.294±0.014	0.442±0.012
15 min	0.317±0.026[b]	0.106±0.002[b]	0.261±0.004	0.331±0.006[b]
30 min	0.249±0.035[b]	0.096±0.006[b]	0.243±0.004[b]	0.281±0.004[b]
60 min	0.174±0.005[b]	0.093±.001[b]	0.219±0.004[b]	0.222±0.016[b]

[a]Activities (umol $NADP^+$ reduced/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

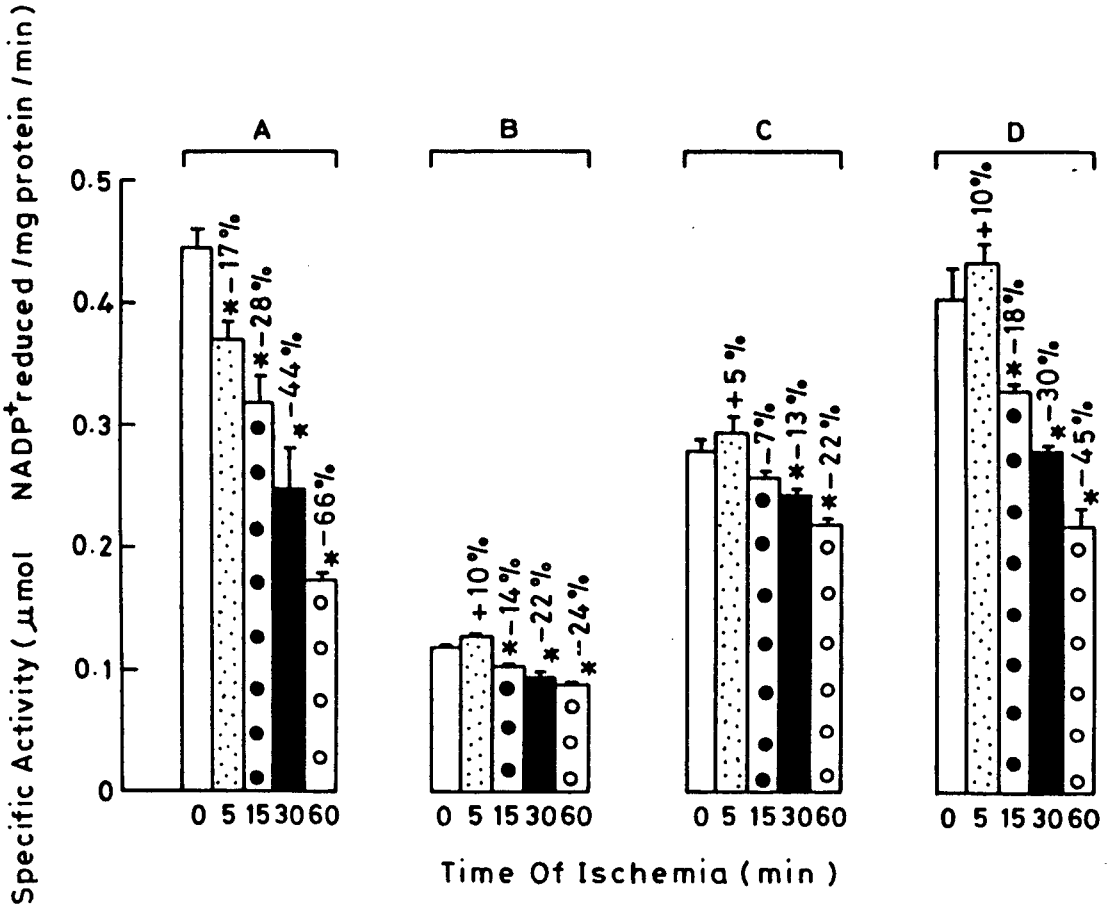


Fig.23: Specific Activity of ME in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min of ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

Table-32: EFFECT OF 5-60 min ISCHEMIA ON THE ACTIVITY OF Na+-K+ ATPase IN THE HOMOGENATES OF MEDULLA, WHOLE CORTEX, SUPERFICIAL, JUXTAMEDULLARY CORTEX.

Time of Ischemia	Medulla	Whole Cortex	Superficial Cortex	Juxtamedullary Cortex
0 min	2.425±0.273[a]	1.897±0.41	1.766±0.064	0.676±0.0014
5 min	1.861±0.297	1.283±0.092	1.840±0.011	0.688±0.001
15 min	1.538±0.215[b]	1.275±0.083	1.400±0.001[b]	0.604±0.0012[b]
30 min	1.300±0.082[b]	1.199±0.124	0.612±0.0012[b]	0.380±0.048[b]
60 min	1.184±0.072[b]	0.775±0.032[b]	0.600±0.001[b]	0.231±0.027[b]

[a]Activities (umol Pi released/mg protein/hr) are expressed as Mean ±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

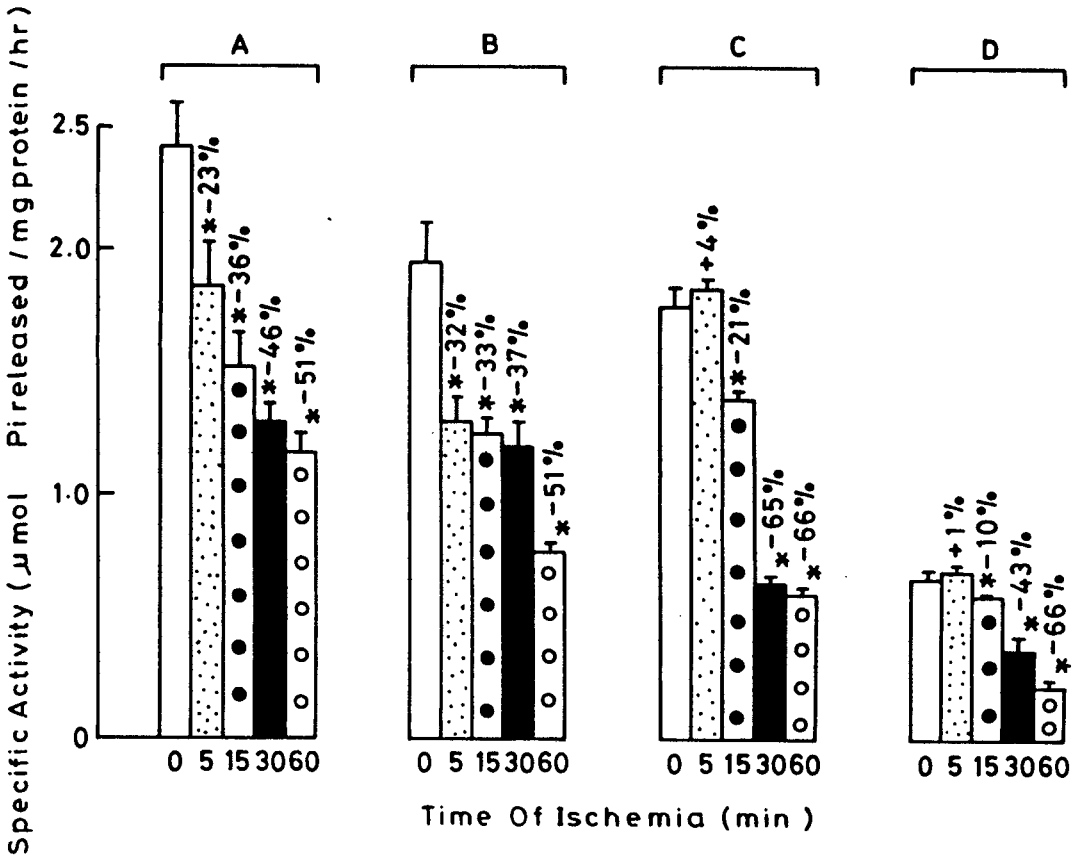


Fig.24: Specific Activity of Na^+/K^+ ATPase in homogenates of (A) Medulla (B) Whole cortex (C) SC-cortex and (D) JMC-cortex after 5-60 min ischemia.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) ~~p<0.05 or higher degree by group t-test.~~

(S_3-subsegments) together with deep PCT, MAL, MCT, PCD and LH. The effect of ischemia after various time intervals was more prominant in either of the kidney tissue for different enzymes. The activity of LDH was increased after 5 min ischemia but only in the JMC. However, the enzyme was greately affected (decrease in the activity) in the SC compared to the JMC after 15, 30 or 60 min ischemia (Table 26; Fig. 18 C & D). The activity of MDH was also lowered to a greater extent in the SC than JMC and the decrease in MDH activity was dependent on the duration of ischemia (Table 27; Fig. 19 C & D). The effect of ischemia on the activities of FBPase and G6Pase in the SC and JMC homogenates was similar to those on the cortical and medullary homogenates. A brief 5 min ischemia caused enhancement in the enzyme activities while they were lowered progressively after 15, 30 and 60 min of ischemia, respectively (Table 28 & 29; Fig. 20 & 21 C & D). The activity of FBPase was similarly decreased both in the SC and JMC homogenates. However, G6Pase activity declined affectively in the SC compared to the JMC homogenates.

The activities of G6PDH and ME were also increased after 5 min ischemia both in the SC and JMC homogenates

(Table 30 & 31), however, the activity of G6PDH increased to much greater extent. Similar effects were observed after 15, 30 and 60 min ischemia in both the SC and JMC homogenates in which the activities declined with increased duration of ischemia (Fig. 22 & 23 C & D). However, after 15, 30 and 60 min ischemia, the activity of ME decreased to greater extent in JMC homogenate compared to SC homogenates (Fig. 23 C & D). The activity of Na^+/K^+ ATPase also decreased due to the ischemia produced for different durations. After 15 and 30 ischemia the effect was apparently more in JMC than in SC homogenates (Table 32; Fig. 24 C & D).

The activity of acid phosphatase (APase) also increased with the time of ischemia, but to a greater extent in JMC than in SC (Table 33).

Effect of blood reflow on the metabolic enzymes activities after 30 min ischemia

The damaging effect of ischemia upto 30 min has found to be reversible (7,60,132) and the blood reflow showed partial recovery in the morphologic and certain functional aspects of the renal cortical proximal tubular cells (7-9,60,132). In the present studies the activities of some BBM marker enzyme were also found to be partially recovered from

Table 33: EFFECT OF 15-60 min ISCHEMIA ON THE ACTIVITY OF ACID PHOSPHATASE IN HOMOGENATES OF SUPERFICIAL AND JUXTAMEDULLARY CORTEX.

Time of Ischemia	SC	JMC
0 min	6.55±0.36[a]	5.78±0.23
15 min	5.77±0.35 (-12%)[c]	5.97±0.23 (+3%)[c]
30 min	7.77±0.26[b] (+19%)[c]	8.17±0.92[b] (+41%)[c]
45 min	9.12±0.58[b] (+39%)[c]	9.57±0.18[b] (+66%)[c]
60 min	13.14±0.38[b] (+105%)[c]	13.00±0.24[b] (+125%)[c]
Contralateral		
15 min	7.19±0.095	6.19±0.075
30 min	6.42±0.31	6.15±0.70
45 min	6.22±0.053	7.35±0.045
60 min	6.135±0.117	5.46±0.36

[a]Activities (umol/mg protein/hr) are expressed as Mean±SEM of three different preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]% change from Control.

ischemic trauma (Table 18-21). Therefore, the effect of the blood reflow was determined on the activities of the metabolic enzymes after 30 min ischemia to observe the changes under similar conditions in the same tissue preparations.

As shown in Fig. 25A (Table 34), the activity of LDH decreased in the cortex (-15%) and medulla (-29%) after 30 min ischemias, 2 min blood reflow. Further blood reflow for 15 min, caused additional decrease of the LDH in the cortex (-23%) but a recovery was seen in the medulla (-8%) compared to control values. After 60 min reflow LDH, activity showed a small recovery (-8%) in the cortex while the enzyme activity in the medulla was fully recovered almost to the control values (Fig. 25A) which were +8% and +51% compared to 2 min reflow in the cortex and the medulla, respectively. The damage occurred due to ischemia and the recovery due to blood reflow appeared to be greater in the medullary LDH activity.

The activity of MDH was similarly lowered by 30 min ischemia and 2 min blood reflow as that of the LDH. However, after 15 min reflow the loss of MDH activity was reduced which was further reduced after 60 min blood reflow (Table

TABLE-34: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF LACTATE DEHYDROGENASE (LDH) IN HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	1.41±0.005[a]	1.80±0.029
2 min	1.20±0.020[b]	1.27±0.017[b]
15 min	1.08±0.005[b,c]	1.66±0.025[c]
60 min	1.30±0.020[c]	1.92±0.045[c]

[a]Activities (umol NADH oxidized/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

TABLE-35: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF MALATE DEHYDROGENASE (MDH) IN HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	8.66±0.44[a]	5.65±0.08
2 min	5.40±0.04[b]	4.26±0.18[b]
15 min	6.23±0.17[c]	4.55±0.01[b]
60 min	6.74±0.05[b,c]	4.70±0.07[b,c]

[a]Activities (umol NADH oxidized/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

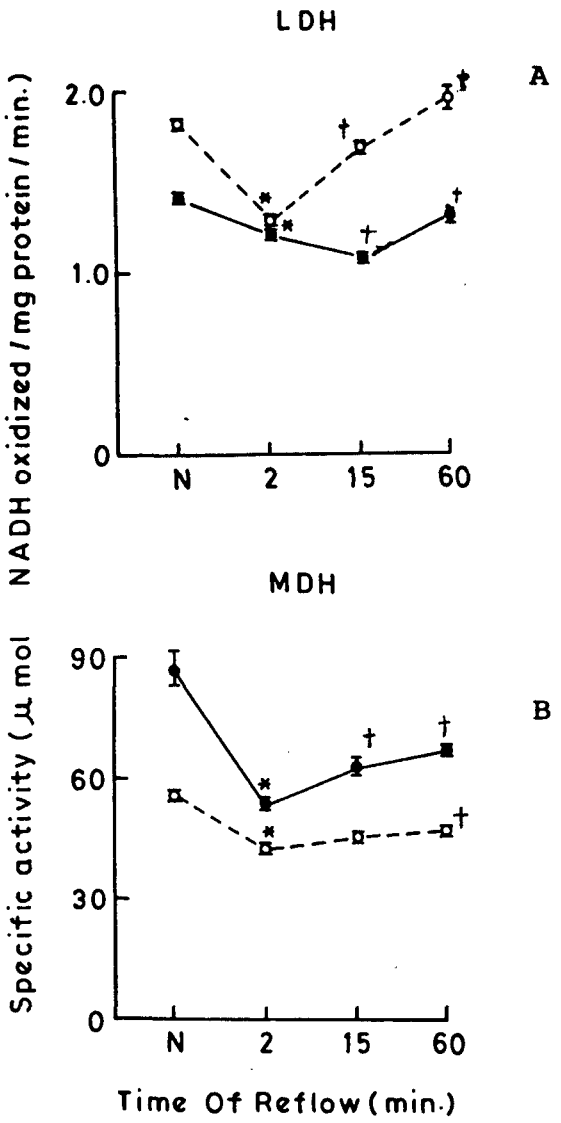


Fig.25: Specific Activities of (A) LDH and (B) MDH in homogenates of Medulla (--o--) and Whole cortex (—●—) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 2 min reflow $p<0.05$ or higher degree by group t-test.

36). However, the activity could not return to the control values. The damage due to ischemia as well as the recovery due to blood reflow was found to be greater in the cortex than the medulla (Fig. 25B).

The activity of the enzymes belonging to gluconeogenesis i.e. FBPase and G6Pase were also lowered after 30 min ischemia and 2 min blood reflow although differentially in the cortex and medulla. They attained the control level activity , however, only after 15 min blood reflow (Fig. 26 A & B; Table 36 & 37) and after 60 min reflow the activities were even greater than the control values both in the cortex as well as in the medulla.

The activity pattern of G6PDH and ME after 30 min ischemia and during blood reflow showed that after ischemia the activity of G6PDH declined to a greater extent in the the medulla while the activity of ME was greately affected in the cortex. However, after 60 min blood reflow the activity of G6PDH was not only restored but even surpassed the control values and the activity of ME almost returned to the control values both in the cortex and the medulla (Table 38 & 39; Fig. 27 A & B).

TABLE-36: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME INTERVALS ON THE ACTIVITY OF FRUCTOSE-1,6-BISPHOSPHATASE (FBPase) IN HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	0.558±0.019[a]	0.178±0.006
2 min	0.500±0.011[b]	0.139±0.001[b]
15 min	0.580±0.006[c]	0.166±0.004[c]
60 min	0.714±0.014[b,c]	0.226±0.010[b,c]

[a]Activities (umol Pi released/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

TABLE-37: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATASE (G6Pase) IN HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	0.168±0.003[a]	0.087±0.001
2 min	0.142±0.002[b]	0.083±0.001[b]
15 min	0.171±0.005[c]	0.089±0.004[c]
60 min	0.175±0.003[c]	0.092±0.001[c]

[a]Activities (umol Pi released/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

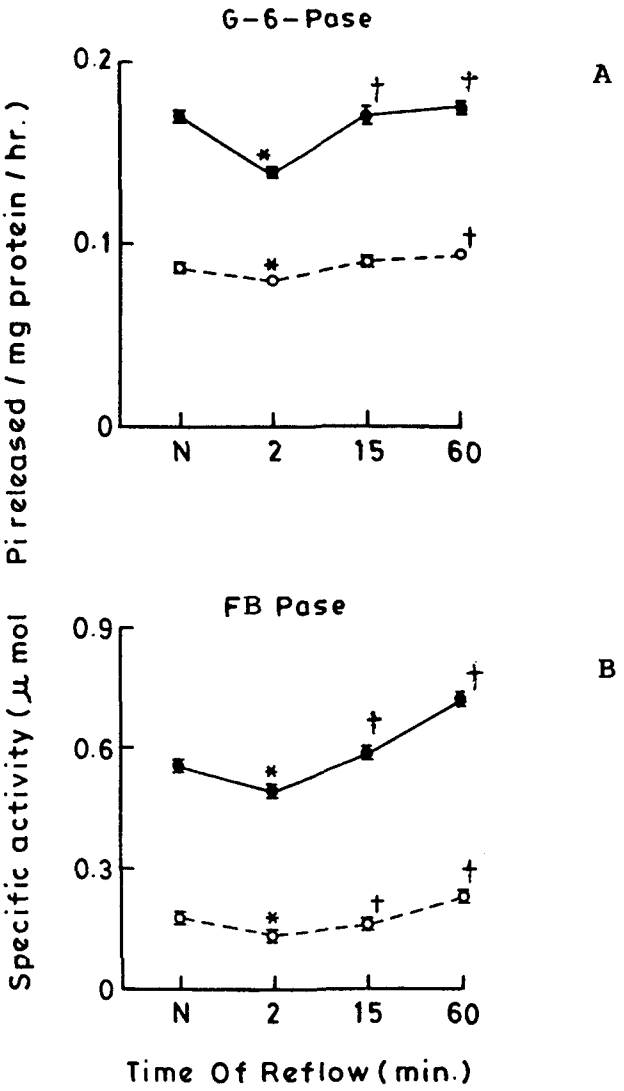


Fig.26: Specific Activities of (A) G6Pase and (B) FBPase in homogenates of Medulla (--o--) and Whole cortex (—●—) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

†Significantly different from 2 min reflow $p<0.05$ or higher degree by group t-test.

TABLE-38: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDRO-GENASE (G6PDH) IN THE HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	0.270±0.015[a]	0.680±0.040
2 min	0.210±0.030[b]	0.385±0.031[b]
15 min	0.255±0.004	0.500±0.001[c]
60 min	0.455±0.015[c]	0.750±0.002[c]

[a]Activities (umol $NADP^+$ reduced/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

TABLE-39: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF MALIC ENZYME (ME) IN THE HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	0.128±0.002[a]	0.462±0.009
2 min	0.091±0.002[b]	0.384±0.011[b]
15 min	0.096±0.001[b]	0.396±0.001[b]
60 min	0.121±0.002[c]	0.462±0.004[c]

[a]Activities (umol $NADP^+$ reduced/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

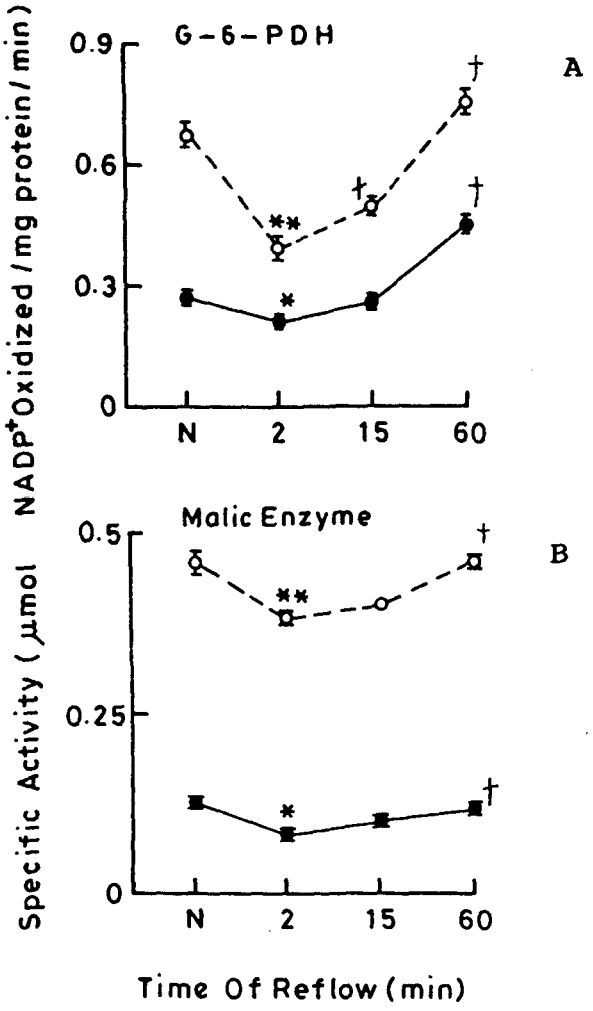


Fig.27: Specific Activities of (A) G6PDH and (B) ME in homogenates of Medulla (--o--) and Whole cortex (—●—) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

†Significantly different from 2 min reflow $p<0.05$ or higher degree by group t-test.

The activity of Na+/K+ ATPase which was decreased due to 30 min ischemia was fully recovered in the cortex after 60 min blood reflow and partially recovered in the medulla (Table 40). The data on the enzymes suggest that blood reflow for 60 min was not only able to reverse the damage caused by 30 min but in most of the enzymes even returned to the control level of activities (Fig. 25-28).

TABLE-40: EFFECT OF 30 min ISCHEMIA AND REFLOW OF DIFFERENT TIME PERIODS ON THE ACTIVITY OF Na^+-K^+ ATPase IN THE HOMOGENATES OF CORTEX AND MEDULLA.

Time of Reflow	Cortex	Medulla
Control	1.158±0.013[a]	1.692±0.006
2 min	0.714±0.007[b]	1.096±0.007[b]
15 min	1.109±0.007[b,c]	1.229±0.012[b,c]
60 min	1.158±0.005[c]	1.325±0.011[b,c]

[a]Activities (umol Pi released/mg protein/min) are expressed as Mean±SEM for three different preparations. Each preparation includes kidneys from 2-3 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 2 min Reflow group $p < 0.05$ or greater degree by group t-test.

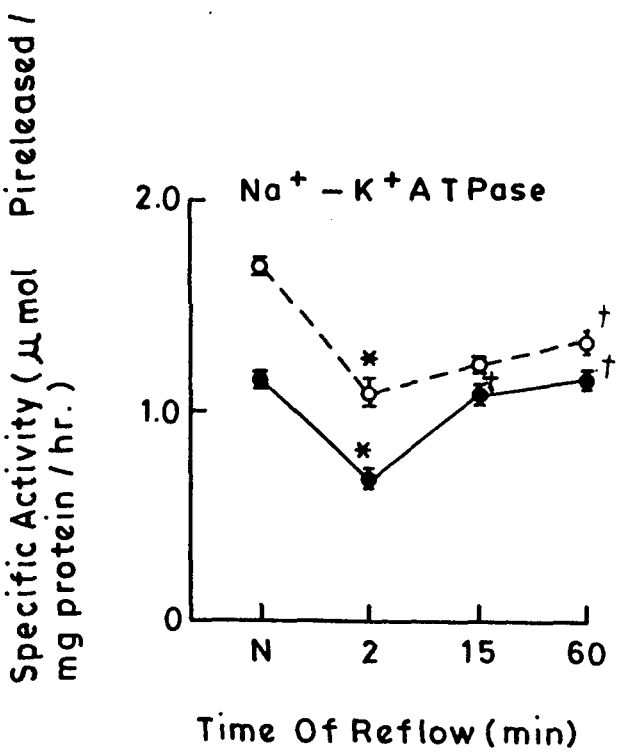


Fig.28: Specific Activity of Na^+/K^+ ATPase in homogenates of Medulla (--o--) and Whole cortex (—●—) after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 2 min reflow $p<0.05$ or higher degree by group t-test.

DISCUSSION - II

The structure and functions of the kidney and its various components in animals and humans have been greately characterized both in physiologic and pathophysiologic conditions (129,210,211). The kidney is consisting of different tissue zones such as cortex, outer and inner medulla which themselves behave rather individual organs (57). Further, "nephron", the structural unit of the kidney is also comprising of various subsegments of distinct structures and specific functions. Intra- and inter-nephronal heterogeneity is also very well understood (212). It is well known that the kidney plays extremely important role in the maintenance of body fluid volume, composition and the pH by virtue of its reabsorptive properties (213). The reabsorption of Na^+ is considered to be the major work function of the kidney (214-216), because the transport of water, other ions and various solutes depends on Na^+ reabsorption directly or indirectly (214-216). The proximal tubule of the renal cortex has been shown to be the major nephron site where majority of ions and solutes including Na^+ are reabsorbed across the luminal brush border membrane mostly by active transport process (46,47,129,157,217,218). The energy required for these transports are usually protracted by the hydrolysis of ATP supplied by various

metabolic activities of the cell (211). A direct relation between the transport of Na^+ and energy yielding metabolic reactions (e.g. glycosis and TCA cycle) concomittent with oxygen-tension (pO_2) or its utilization in various kidney tissue zones or cells has been demonstrated (50-55). The transport of several solutes dependent on Na^+ reabsorption are also regulated at the BBM site under various physiologic, pathophysiologic and pharmacologic conditions (47,129,218-221).

It has been shown that ischemia decreases renal ATP content and causes alterations in the activities of certain metabolic enzymes belonging to carbohydrate metabolism and in the levels of various metabolic substrates in renal tissues (6,112,126-128,222).

The existence of the major biosynthetic and catabolic pathways of intermediary metabolism especially for carbohydrates in the adult mammalian kidney has been known a long time (50,52). However, the enzyme activities of above pathways were not studied in detail during ischemic and toxic ARF or during the recovery period. It is known that glycolysis preferably occurs in the medulla (57,113-115)

while oxidative metabolism (e.g. TCA cycle) is more prevalent in the renal cortex (57,115). In the present study the activities of certain metabolic enzymes were simultaneously determined in the homogenates of cortex and outer medulla and/or of superficial (SC) and juxtamedullary cortex (JMC) isolated from rats subjected to ischemic injury for various duration (e.g. 5 min-60 min). As described in the "Results", a brief 5 min ischemia caused a rapid increase in the activities of LDH both in the cortex and medulla (Table 26; Fig. 18A & B). The activity of FBPase was also increased under similar conditions of 5 min ischemia indicating that glycolysis might be occurring at rapid rate (Table 28; Fig. 20 A & B). While the increase in LDH was similar in both cortex and medulla, the activity of FBPase was increased to greater extent in the cortex than in the medulla resulting in higher production of lactic acid (112). Similar accumulation of lactate was also obtained after a brief ischemia of 2-5 min by Bastin et al. (112), although LDH activity or the actual rate of glycolysis were not determined. When the ischemia was prolonged (15-60 min), the activity of LDH declined both in the cortex and the medulla to similar extent after 15 min ischemia. The activity was continued to decrease further, after 30 or 60 min ischemia

(Table 26; Fig. 18A & B). The activity of FBPase was also lowered after 15, 30 or 60 min ischemia but to much greater extent in the medulla than in the cortex (Table 28; Fig. 20 A & B). The initial increase of LDH or FBPase activity after 5 min ischemia appeared to be an adaptive change due to quick stoppage of the blood flow and due to the reduced oxygen supply causing a relatively more anerobic condition as has been suggested also in earlier studies (60,112,126,127). However, the decline in the activities after 15, 30 or 60 min ischemia appeared to be due to reduced availability of substrates and the active enzyme molecules themselves.

The activity of MDH, however, was differentially affected by ischemia produced for various durations (Table 27; Fig. 19 A & B). The activity of MDH lowered at all time points of ischemia (5 min-60 min) and it was in proportion to the duration of ischemia. This observation was in agreement with the results of the earlier studies in which the activities of MDH and ICDH were found to be lowered by ischemic injury (126). Other studies have demonstrated a decrease in the contents of a-KG, pyruvate, OAA and glutamate, substrates for TCA cycle enzymes after brief or

prolonged ischemia (86,87) indicating an overall decline in the TCA cycle pathway. These observations together with the present observations guardedly suggest that in ischemic injury the oxidative metabolism is affected to limit the production of ATP mainly due to the disrupted O_2 supply. In the kidney, cortical MDH appears to be greatly affected than the medullary enzyme (Table 27; Fig. 19 A & B).

The activities of the enzymes involved in the synthesis of glucose e.g. G6Pase (Fig. 21 A & B) and FBPase (Fig. 20 A & B) were significantly increased similar to LDH after a brief 5 min ischemia. However, the increase in the activities was more prominent in the cortex compared to the medulla. In renal cortex, after 15 min or 30 min ischemia FBPase activity declined in comparison to 5 min ischemia, but was not significantly different from sham-operated control rats. However it was significantly lowered in the medulla. After 60 min ischemia FBPase activity also declined in the cortex (-18%) but in the medulla it was lowered significantly (-50%). The effect on the G-6Pase activity after 15, 30, 60 min ischemia was similar to that of FBPase and medullary enzyme was significantly and greately reduced in comparison to the cortical enzyme. The results clearly

demonstrate a differential effect of ischemia on the above enzymes in the cortex and medulla and the effect was dependent on the duration of ischemia. The initial increase in the activity of enzymes after 5 min ischemia appeared to synthesize enough glucose from non-carbohydrate substrates to maintain a positive energy balance as indicated by the increased activities of LDH (glycolysis) and MDH (TCA cycle) especially in the cortex for transport activities.

Ischemia caused a mixed effect on the activities of G6PDH (Table 30; Fig. 22 A & B) and ME (Table 31; Fig. 23 A & B), both involved in the production of NADPH required for many biosynthetic pathways. A brief 5 min ischemia caused a small increase in ME activity only in the cortex otherwise both the enzymes in both the tissues (cortex and medulla) were significantly declined in proportion to the duration (15-60 min) of ischemia. The effect was always greater in the medulla than in the cortex.

When the above metabolic enzymes were determined in the superficial (SC) and juxtamedullary cortex (JMC) after different duration of ischemia, the activities of various enzymes were differentially affected in SC and JMC tissues

compared to cortex and medulla. In this case only outer most half part of the cortex was taken as SC-tissue while JMC included the lower half of the cortex together with the outer-stripe of outer medulla (see "Methods" for details). It is well known that various nephronal subsegments are differentially distributed in SC and JMC tissues (223). It has been demonstrated both by morphologic and biochemical studies that SC tissue contains predominantly early proximal convoluted tubule (S_1 and S_2-subsegments) with distal nephronal subsegments such as DCT and CCT, while JMC tissue is enriched with proximal straight tubule (PST, pars recta, S_3-subsegments) of both superficial and deep nephrons together with deep PCT and other distal nephronal subsegments such as MCT, MAL and thin segments of LH (113,165). The activities of various metabolic enzymes have been determined and were found to be differentially distributed in these nephronal subsegments (113). Because of the above fact the effect of ischemia was observed in SC and JMC tissues. The activities of both LDH (Table 26; Fig. 18 C & D) and MDH (Table 27;Fig. 19 C & D) declined progressively with the increased duration of ischemia with the exception of LDH activity which was increased with 5 min ischemia. At

all time points of ischemia the decrease in activities of LDH and MDH were much greater than previously observed in the cortex or medulla and the decrease was more profoundly greater in the SC than JMC. This correlates with the fact that O_2-tension is certainly higher in the superficial than in the deep cortex (108-111) and in superficial region PCT and DCT showed a higher oxidative metabolic activity. Thus depleted O_2-tension due to ischemia might have resulted in decreased activities of above enzymes. As a result ATP will not be produced in sufficient amount and would result in the decreased transport functions.

The gluconeogenic activity as apparent from the activities of FBPase (Table 28; Fig. 20 C & D) and G6Pase (Table 29; Fig. 21 C & D) was also differentially altered by ischemia in the SC and JMC tissues compared to whole cortex and medullary data (Table 28 & 29). A brief 5 min ischemia increased the activities of FBPase and G6Pase while prolonged 15, 30, 60 min ischemia lowered the activities significantly. While the activity of FBPase was evenly affected both in the SC and JMC-tissues, the activity of G6Pase was greatly altered in the SC than JMC homogenates.

Similarly the activities of G6PDH and ME in SC and JMC-

tissues were differentially affected by the ischemia of various durations. A short 5 min ischemia caused a significant increase in the activity of G6PDH (Table 30; Fig. 22 C & D) both in the SC (+63%) and JMC (+42%) while prolonged ischemia produced a decrease however, to a similar extent both in the SC and JMC homogenate. On the other hand ME activity (Table 31; Fig. 23 C & D) was slightly increased after 5 min ischemia but significantly and progressively decreased with respect to the duration of ischemia and the affect was much greater in the JMC than in SC homogenates.

The effect of blood reflow after ischemia on the metabolic enzyme activities was determined in 30 min ischemic rats. As the effect of ischemia on the enzymes was different, blood reflow also affected the enzymes differentially in CH and MH. Blood reflow for 15 min caused a further decrease of LDH in the cortex while medullary LDH showed a sign of recovery. Prolonged reflow for 60 min resulted in the complete recovery of LDH in the medulla, the cortical enzyme was only slightly improved (Table 34; Fig. 25 A). The activity of MDH which was lowered by ischemia both in CH and MH showed small recovery both in CH and MH only after 15 min reflow. However the activity was

significantly improved after 60 min reflow but the activity of MDH was still lower than control values (Table 35; Fig. 25 B). the effect of reflow was greater in medulla than cortex.

The activities of G6Pase and FBPase were recovered to control values both in CH and MH only after 15 min reflow and increased higher than control values after 60 min reflow (Table 36 & 37;Fig. 26 A & B). The activities of G6PDH and ME which were lowered greatly due to ischemia in the medulla and cortex respectively, were also recovered completely after 60 min of blood reflow (Table 38 & 39;Fig. 27). Similarly the activity of Na^+/K^+ ATPase was completely recovered in the cortex but partially in the medulla after 60 min reflow (Table 40; Fig. 28).

The results clearly indicate a differential effect of both ischemia and reflow on the cellular enzyme activities, although under similar conditions the activities of BBM enzymes were further decreased after 15 min reflow and only partially restored even after 120 min reflow.

RESULTS & DISCUSSION
PART III

Transport properties of renal PT-BBMV during ischemia and reflow

It has been known that ischemia causes alterations in the renal functions by affecting mainly cellular metabolism of the proximal tubule and the structural integrity of its brush border membrane metabolism. However, the effect of ischemia on the BBM transport of Pi was never studied, the chief contender to be involved in the restoration of the energy required for such transports across the BBMV in the proximal tubules. In the present studies, effect on transport of Pi together with the transport of D-glucose and/or L-proline were determined under (i) ischemic cell injury, (ii) repairing by reflow, (iii) protection or prevention by certain dietary and hormonal stimuli. It has been observed that ischemia causes alterations in the membrane fluidity due to changes in the lipid composition (136-138). In the present study 15-60 min ischemia also induced specific changes in BBM phospholipids and cholesterol contents as shown in Table 41.

a) Effect of ischemia on the transports of Pi and L-proline :

As described in the "Methods", ischemia was produced by

TABLE-41: EFFECT OF 15-60 min ISCHEMIA ON PHOSPHOLIPIDS, CHOLESTEROL, and C/P Ratio OF BBMVs ISOLATED FROM WHOLE CORTEX

Time of Ischemia	Phospholipids (P) (mg/mg protein)	Cholesterol (C) (mg/mg protein)	C/P ratio
0 min	0.175±0.020[a]	0.098±0.006	0.571±0.039
15 min	0.156±0.026	0.082±0.008	0.551±0.072
30 min	0.127±0.010[b]	0.061±0.011[b]	0.504±0.110
60 min	0.186±0.013[c]	0.079±0.003	0.433±0.039[b]

[a]Values are Mean±SEM of three different BBMV preparations. Each preparation includes kidneys from 5-6 rats in each group.

[b]Significantly different from Control (0 min) $p < 0.05$ by group t-test.

[c]Significantly different from 30 min Ischemia $p < 0.05$ by group t-test.

occlusion of the left renal artery for different time periods e.g., 15, 30, and 60 min. To study the effect of ischemia, the blood was reflown for a brief 2 min to perfuse the renal tissue after specified time periods of ischemia. The kidneys from control and ischemic rats (both ischemic and contralateral) were harvested and the brush border membranes were prepared by Mg^{++} precipitation as described in "Methods". The transports of Pi and L-proline were determined by microfiltration technique using ^{32}Pi, and L-[^{3}H]-proline as radiotracers (see "Methods" for detail). The results are summarized in Tables 42-45 (Fig. 29-31). As shown in Table 42 (Fig. 29) Na-gradient dependent (Na_o > Na_i) concentrative uphill transport of Pi significantly decreased in the ischemic rats after 15 min of ischemia determined at all early time points (e.g. 5s, 20s and 40s) compared to non-ischemic sham- operated control rats. However, the uptake of ^{32}Pi was not significantly affected by ischemia at 120 min after equilibration. The uptake of Na^{+}-dependent ^{32}Pi after 30 min ischemia was further declined at 5s only and not at 20s or 40s. Similarly after 60 min ischemia the decrease in ^{32}Pi transport was attenuated. The equilibrium uptakes of ^{32}Pi at 120 min was lowered after 60 min ischemia although not significantly.

Table 42: EFFECT OF 15-60 min ISCHEMIA ON Na+GRADIENT-DEPENDENT ^{32}Pi UPTAKE BY BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Ischemia	5 sec	20 sec	40 sec	120 min
0 min	584.64±11.81[a] [117%][d]	1255.20±11.67 [366%][d]	2154.10±85.83 [699%][d]	269.40±33.47
15 min	393.43±15.43[b](-33%)[c] [40%][d]	695.26±63.52[b](-45%)[c] [148%][d]	1123.84±120.85[b](-48%)[c] [300%][d]	280.58±15.94
30 min	304.48±31.98[b](-48%)[c] [20%][d]	806.04±24.50[b](-36%)[c] [217%][d]	1484.01±142.01[b](-31%)[c] [485%][d]	253.58±20.37
60 min	345.33±18.68[b](-91%)[c] [59%][d]	829.44±17.14[b](-34%)[c] [283%][d]	1287.35±197.33[b](-40%)[c] [494%][d]	216.55±12.27
Contralateral				
15 min	739.69±48.97 [67%][d]	1477.43±19.77 [234%][d]	2135.56±17.83 [383%][d]	441.94±26.52
30 min	710.18±21.13 [31%][d]	1138.21±91.44 [109%][d]	2547.02±63.60 [368%][d]	544.19±23.05
60 min	563.18±13.00 [26%][d]	1502.82±94.98 [237%][d]	2135.72±211.37 [379%][d]	446.14±30.80

[a]Uptake (pmol/mg protein)is expressed as Mean±SEM for four different BBMV preparations.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Values in paranthesis are % decrease from Control values..

[d]Values in square brackets are % upshoot from the respective equilibrium uptake (120 min) values.

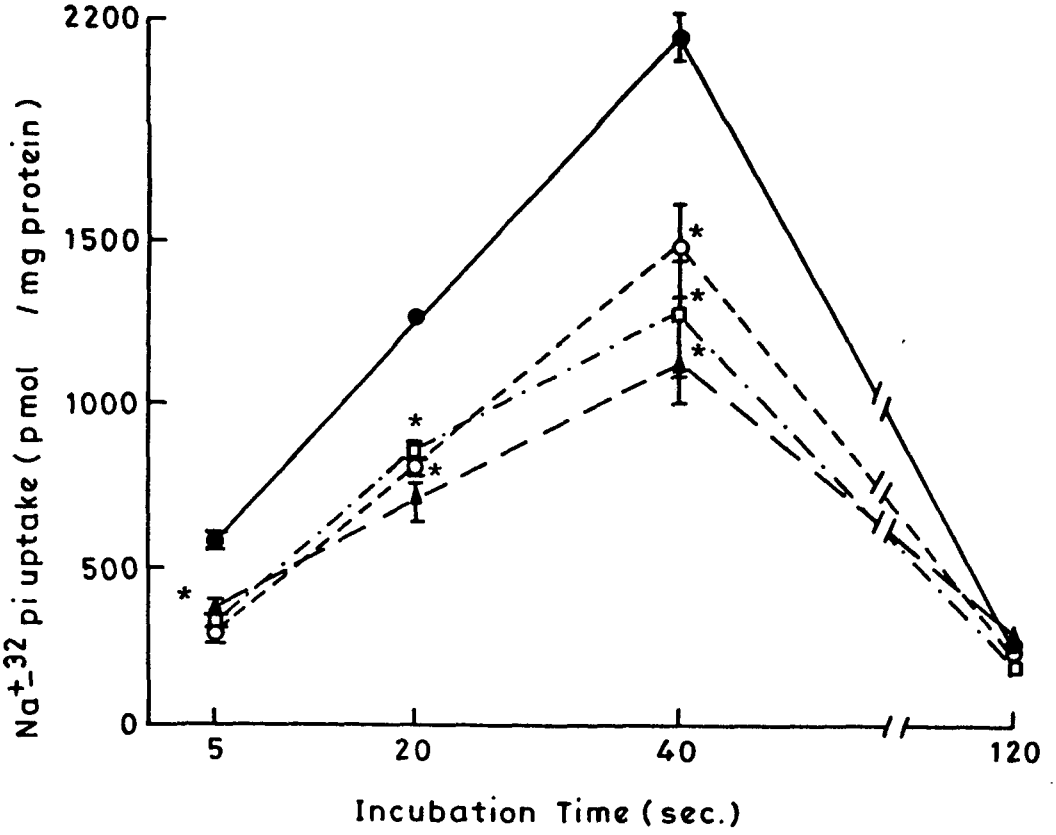


Fig.29: Time course of Na^{+}-gradient dependent ^{32}Pi uptake by BBMV(s) isolated from whole cortex of Control (—●—), and ischemic 15 min (--▲--), 30 min (--o--) and 60 min (—·—□—·—) rats.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

The transport of 32Pi in the presence of a Na+-gradient ($Na_o > Na_i$) was also determined in the BBMV(s) prepared from the contralateral kidneys. Preliminary experiment showed that Na-dependent uptake of ^{32}Pi was not different in the BBMVs when isolated from left or right kidney of control rats and therefore BBMV(s) were preparated by pooling both the kidneys in the sham-operated control groups. A compensatory increase in Na-gradient uptake of ^{32}Pi was observed in the BBMV(s) isolated from contralateral kidneys compared to ischemic kidneys of ischemic rats. The increase not only was observed in the uphill transport phase (5S-40S) but also at the equilibrium (120 min) period. The increase in the transport was more apparent after 15 or 30 min ischemia than after 60 min ischemia where the effect of ischemia was attenuated in the ischemic kidney (Table 42).

Since it has been known that ischemia causes much damage to the proximal tubular epithelia including the microvillar membrane and that the epithelia becomes leaky, the uptake of Pi was also determined in the absence of a Na-gradient (NaCl is replaced by KCl (Ko > Ki) in the incubation medium). The uptake of ^{32}Pi in the absence of Na^+-gradient was not significantly different after 15 min

Table 43: EFFECT OF 15-60 min ISCHEMIA ON Na+-INDEPENDENT ^{32}Pi UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Ischemia	30 sec	120 min
0 min	221.10±21.26[a]	476.36±32.00
15 min	187.36±21.00	518.56±46.70
30 min	414.07±26.93[b]	683.06±32.70[b]
60 min	507.30±32.16[b]	712.24±11.39[b]
Contralateral		
15 min	219.24±14.86	411.17±28.32
30 min	180.91±10.36	465.28±30.19
60 min	240.59±18.79	487.42±44.37

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM for four different BBMV preparation.

[b]Significantly different from Control (0 min) values $p < 0.05$ or greater degree by group t-test.

Table 44: EFFECT OF 15-60 min ISCHEMIA ON NET Na GRADIENT DEPENDENT ^{32}Pi UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX AT 30s INCUBATION.

Time of Ischemia	NaCl (100mM)	KCl (100mM)	Net Uptake (Na-K)
0 min	1704.68±38.75[a]	221.10±21.26	1483.58±25.05
15 min	909.55±61.46[b] (-47%)[c]	187.36±21.00 (-15%)	758.86±33.23[b] (-49%)
30 min	1145.02±55.03[b] (-33%)	414.17±26.93[b] (+87%)	730.95±32.78[b] (-51%)
60 min	1058.40±71.49[b] (-38%)	507.30±32.16[b] (+129%)	551.10±41.46[b] (-63%)

[a]Uptake (pmol/mg protein/30s)is expressed as Mean±SEM for four different BBMV preparation.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Values in paranthesis are % change from Control (0 min) values.

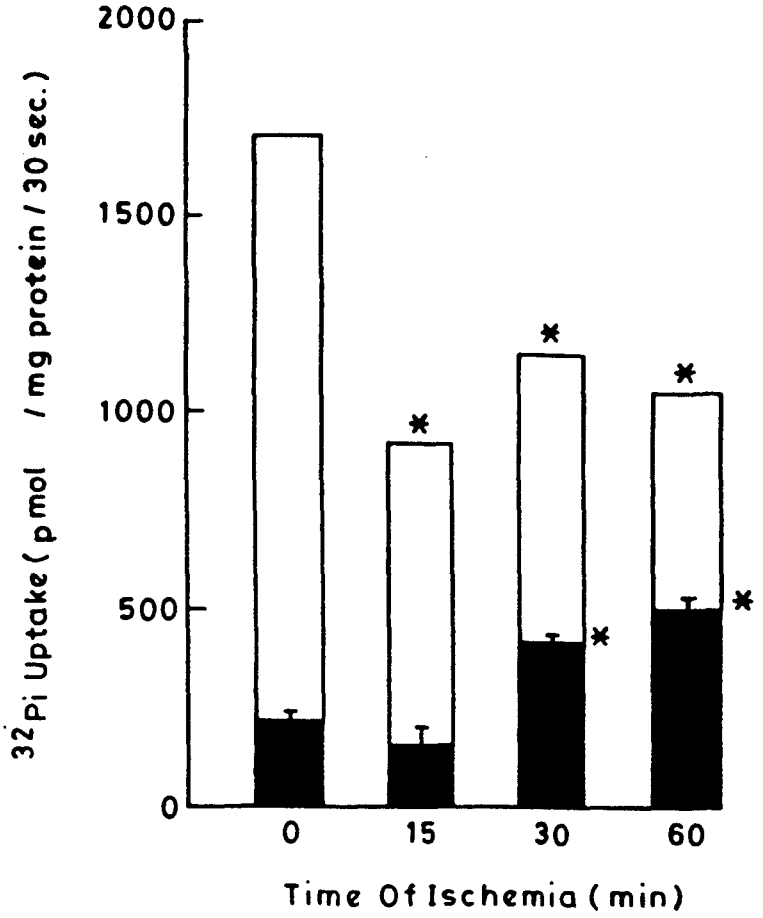


Fig.30: Net Na^+-gradient dependent (□) ^{32}Pi uptake by BBMV(s) isolated from whole cortex after 15-60 min ischemia.

Values are represented as Mean ± SEM for four different preparations

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

ischemia both at 30s and 120 min (Table 43). However, after 30 and 60 min of ischemia the uptake was significantly increased compared to control rats both at 30s and 120 min (Table 43). This indicate that 30-60 min ischemia may have caused alterations in the BBMV(s) to increase diffusional uptake capacity. Na-independent uptake in the contralateral kidney of ischemic rats was not different. Due to these discrepencies net Na^+-dependent uptake (uptake in the presence of Na-gradient - uptake in the absence of Na-gradient) at 30s was also calculated as summarized in Table 44 (Fig. 30). The results indicate that ischemia causes a progressive decline in the net Na^+-dependent uptake of ^{32}Pi in proportation to the duration of ischemia.

In the same preparation of BBMV(s) isolated from whole cortex, the transport of L-[^{3}H]-proline was also significantly decreased after each duration of ischemia (Table 45). The Na-gradient dependent ($Na_o > Na_i$) uptake of L-proline at initial 15s was significantly decreased (-21%) after 15 min ischemia. The uptake was further declined gradually after 30 min (-46%) and 60 min (-50%) ischemia as well. However, the uptake of L-proline at equilibrium (120 min) was not significantly decreased after 15 or 30 min

Table 45: EFFECT OF 15-60 min ISCHEMIA ON L-[3H]-PROLINE UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	Na^+-gradient-dependent		Na^+-gradient-independent	
	15 sec	120 min	15 sec	120 min
0 min	140.07±3.10[a] [47%][d]	73.86±5.64	10.32±0.26	27.97±3.33
15 min	109.84±2.20[b](-21%)[c] [43%][d]	62.65±10.88	8.37±0.15	36.45±1.56
30 min	75.74±7.25[b](-46%)[c] [27%][d]	55.02±7.40	8.51±0.56	27.74±4.01
60 min	70.10±1.40[b](-50%)[c] [33%][d]	46.67±7.82[b]	8.04±1.10	42.83±7.52
Contralateral				
15 min	129.35±7.78 [60%][d]	51.83±3.99	6.18±0.95	31.46±3.41
30 min	110.54±8.40 [40%][d]	66.45±2.74	5.38±0.84	37.16±2.39
60 min	129.10±5.81 [40%][d]	77.34±4.58	7.94±1.38	40.96±3.64

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM for three different BBMV preparations.

[b]Significantly different from Control (0 min) $p < 0.05$ or greater degree by group t-test.

[c]Values in paranthesis are % change from Control.

[d]Values in square brackets are % upshoot from the respective equilibrium (120 min) values.

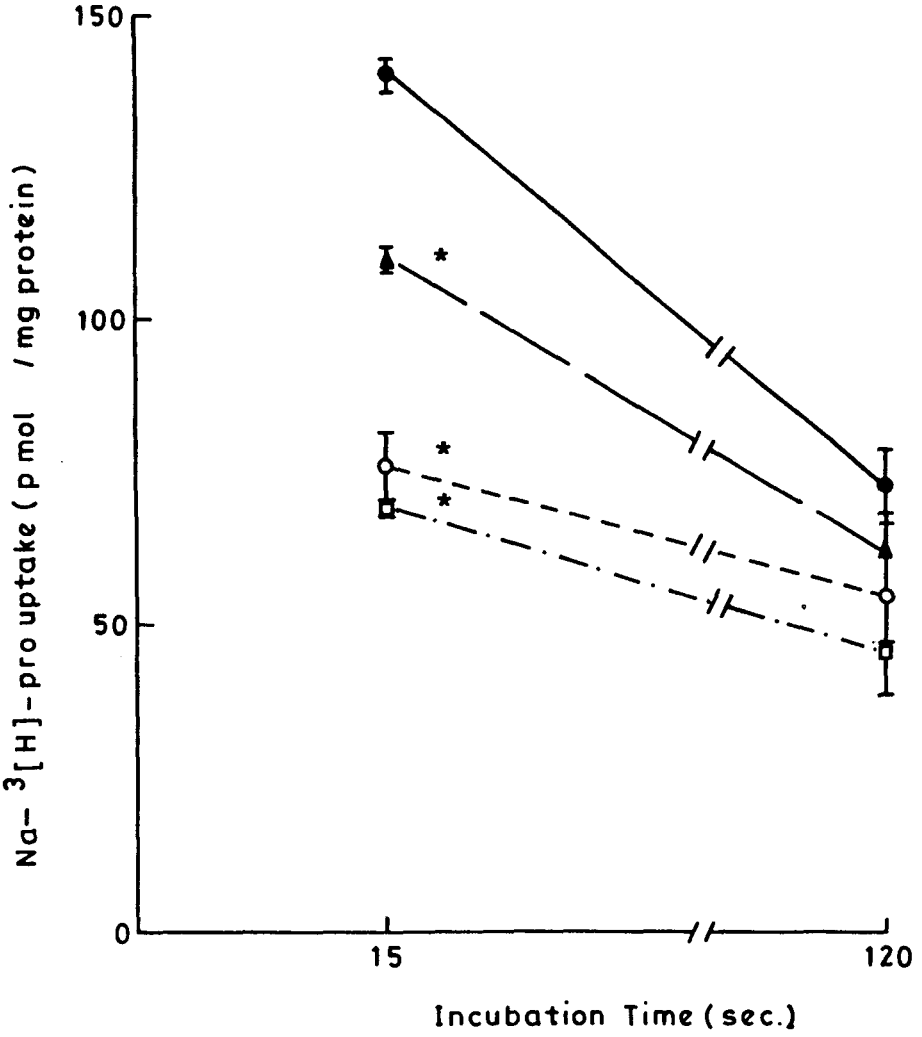


Fig.31: Time course of Na^{+}-gradient dependent L-[^{3}H]-pro uptake by BBMV(s) from whole cortex of Control, (—●—), and ischemic 15 min (—▲—), 30 min (--o--) and 60 min (—·-□-·—) rats.

Values are represented as Mean ± SEM for four different preparations.

*Significantly different from Control (0 min) $p<0.05$ or higher degree by group t-test.

ischemia but declined after 60 min ischemia similar to that of Pi transport (Fig. 31). The Na^+-gradient uptake of L-proline in BBMV(s) from contralateral kidney, unlike Pi transport, was significantly not different from control values both at 15s and 120 min (Table 45). The Na^+-independent uptake of L-proline in the presence of K^+ gradient (Ko > Ki) was also not altered by ischemia both at 15s and 120 min. Similar uptakes of L-proline at 120 min both in the presence and absence of Na-gradient suggest a similar intra-vesicular volume of BBMV(s) isolated from control and ischemic rats.

b) Effect of ischemia and reflow combine on the transport properties of renal proximal tubular BBMV(s) :

i) Effect of 30 min ischemia and 120 min reflow on the transports of ^{32}Pi D-[^3H] glucose and L-[^3H]-proline:

It is well established and also aparent from the present study so far that the damage caused by ischemia to the proximal tubules is dependent on the duration of ischemia. It has been demonstrated by morphologic and other studies that damage caused by the ischemia to the kidney of shorter duration (2-30 min) was reversible and could be restored partially or fully by the blood reflow for several

hours (7-9,60). However, the damage caused by the ischemia of longer duration ($\geq$ 60 min) were usually irreversible and could not be restored even after blood reflow for several days or months (7-9,60,132). This information is of utter importance to enhance further knowledge regarding the mechanism of the pathogenesis of ARF as well as for its prevention or treatment. The following experiments were carefully designed to study the effect of ischemia and subsequent blood reflow to ascertain whether transport properties of the proximal tubular membranes are also altered reversibly and irreversibly under short or prolonged ischemic conditions. The ischemia was produced for either 30 min or 60 min duration and then the blood was reflown for 2, 15, 60, and/or 120 min. BBMV(s) were isolated from whole cortex and the transports of ^{32}Pi, D-[^{3}H]-glucose and/or L-[^{3}H]-proline were determined in the above conditions. As observed in the previous section, after 30 min ischemia and 2 min reflow Na^{+}-gradient dependent transport of ^{32}Pi was significantly decreased (35-40%) when determined in early time phase i.e. 10s or 30s. The reflow of blood for 15 min resulted in the further lowering (~55%) of the initial uptake of ^{32}Pi both at 10s and 30s. However, the blood reflow for 60 min caused a reversal in the

Table 46: EFFECT OF 30 min ISCHEMIA AND REFLOW ON 32Pi UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	Na^+-gradient dependent			Na^+-gradient independent
	10 sec	30 sec	120 min	30 sec
Control	399.67±13.81[a]	568.93±31.62	157.54±18.72	68.07±4.51
2 min	240.59±5.15[b] (-40%)[d]	366.00±9.22[b] (-36%)[d]	142.75±3.90	67.03±2.85
15 min	170.20±10.02[b] (-57%)[d]	256.18±22.73[b] (-55%)[d]	189.66±39.90	71.71±6.55
60 min	210.63±16.39[c] (+24%)[e]	338.03±9.59[c] (+32%)[e]	176.76±41.20	64.20±5.65
120 min	267.24±11.09[c] (+57%)[e]	458.94±25.87[c] (+79%)[e]	163.54±22.34	63.72±2.25

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM of three different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

[d]Values are % change from Control.

[e]Values are % change from 15 min Reflow group.

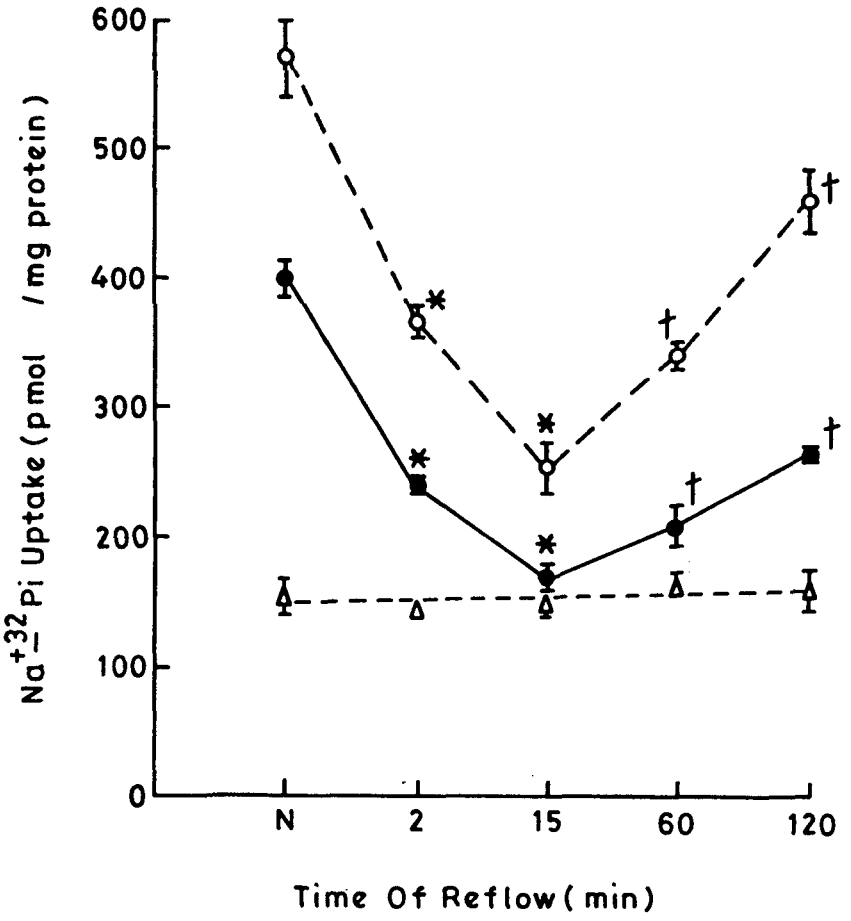


Fig. 32: Time course of Na^{+}-gradient dependent ^{32}Pi uptake by BBMV(s) from whole cortex after 30 min ischemia and reflow at 10s (—●—), 20s (--o--) and 120 min (-△-) incubation.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

decreased uptake of 32Pi induced by 30 min ischemia compared to 15 min RF. The uptake ^{32}Pi was significantly increased (24-32%) in 60 min RF rats. Na^+-gradient dependent uptake of ^{32}Pi was profoundly increased (60-80%) after 2 hr reflow compared to 15 min RF (Fig. 32). The results clearly indicate that the effect of 30 min ischemia on ^{32}Pi uptake was indeed reversible and that blood reflow for 120 min resulted in the restoration of the transport between 70-80% of the control values. The results further indicate that Na^+-gradient dependent equilibrium uptake at 120 min (Table 46, Fig. 32) and Na^+-independent uptake determined at 30s were not changed significantly during 30 min ischemia and 2 hr post ischemic blood reflow (Table 46). Under similar experimental conditions in the same BBMV(s) preparations the Na^+-gradient dependent uptake of D-[^{3}H]-glucose (Table 47) and L-[^{3}H]-proline (Table 48) were also decreased in 30 min-(2 min RF) ischemic BBMV(s) compared to the control BBMV(s) preparation. The uptakes were further decreased after 15 min reflow similar to that of ^{32}Pi transport under the same conditions. The blood-reflow for 60 or 120 min showed recoveries in the transport of both D-glucose and L-proline in the early phase just the same way as ^{32}Pi transport did (Fig. 33 & 34). However, no significant difference was

Table 47: EFFECT OF 30 min ISCHEMIA AND REFLOW ON D-[3H]-Glu BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	Na^+-gradient-dependent		Na^+-gradient-independent	
	30 sec	120 min	30 sec	120 min
Control	96.64±5.95[a]	40.83±9.44	14.18±2.36	43.51±6.83
2 min	74.36±3.36[b] (-23%)[d]	44.09±3.82	11.87±1.47	45.26±1.14
15 min	60.58±4.41[b] (-37%)[d]	44.77±2.40	15.98±0.60	40.14±1.72
60 min	81.32±5.83[c] (+34%)[e]	40.06±1.41	13.50±2.35	41.75±6.73
120 min	91.01±2.03[c] (+50%)[e]	41.77±3.65	16.61±3.33	49.43±12.49

[a]Uptake (pmol/mg protein)is expressed as Mean±SEM of three different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

[d]% change from Control.

[e]% change from 15 min Reflow group.

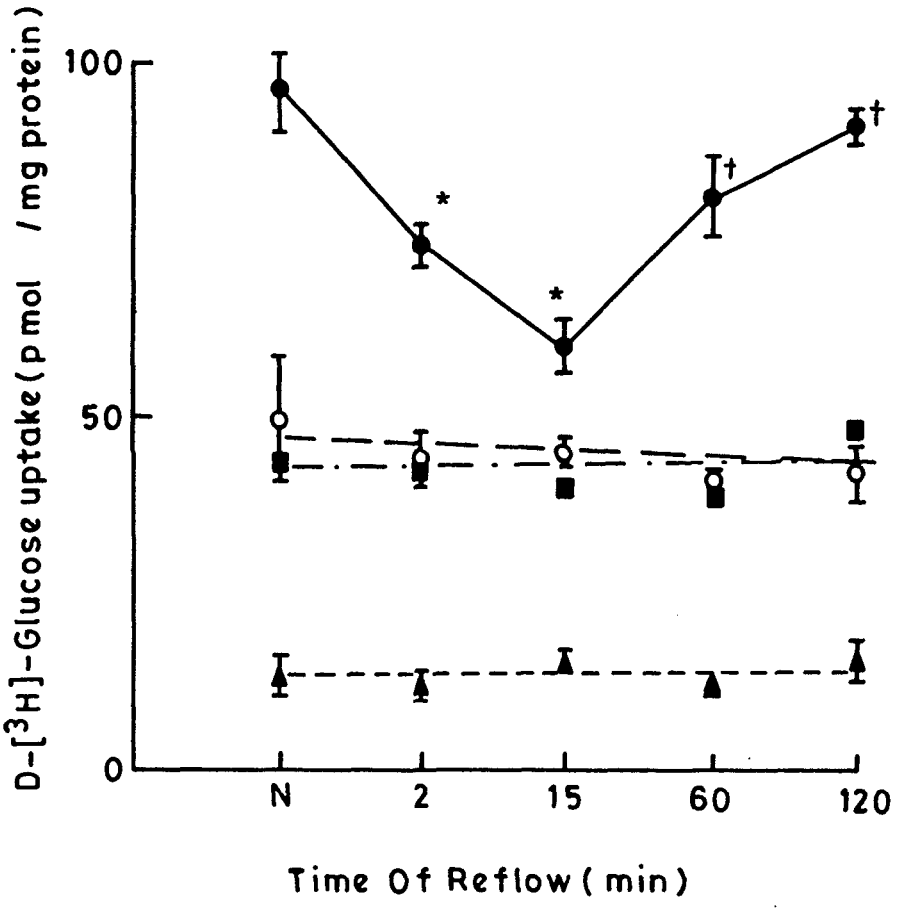


Fig.33: Time course of D-[^{3}H]- Glu uptake in presence of Na^+-gradient (—●— , 30s and --o--, 120 min incubation) and in absence of Na^+-gradient (---Δ--- 30s, and —•■•—, 120 min incubation) by BBMV(s) from whole cortex at 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

Table 48: EFFECT OF 30 min ISCHEMIA AND REFLOW ON L-[3H]-pro UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	Na^+-gradient-dependent		Na^+-gradient-independent	
	30 sec	120 min	30 sec	120 min
Control	53.33±1.84[a]	20.07±1.39	6.13±1.06	23.06±2.40
2 min	37.37±1.40[b] (-30%)[d]	22.96±2.23	7.42±1.47	26.83±0.94
15 min	28.25±1.80[b] (-47%)[d]	21.87±2.27	6.73±1.55	20.30±1.61
60 min	42.19±4.16[c] (+50%)[e]	26.10±3.67	6.20±1.03	19.45±1.58
120 min	51.27±2.38[c] (+81%)[e]	27.74±2.14	7.11±1.00	22.13±2.75

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM of three different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by Control t-test.

[d]% change from Control.

[e]% change from 15 min Reflow group.

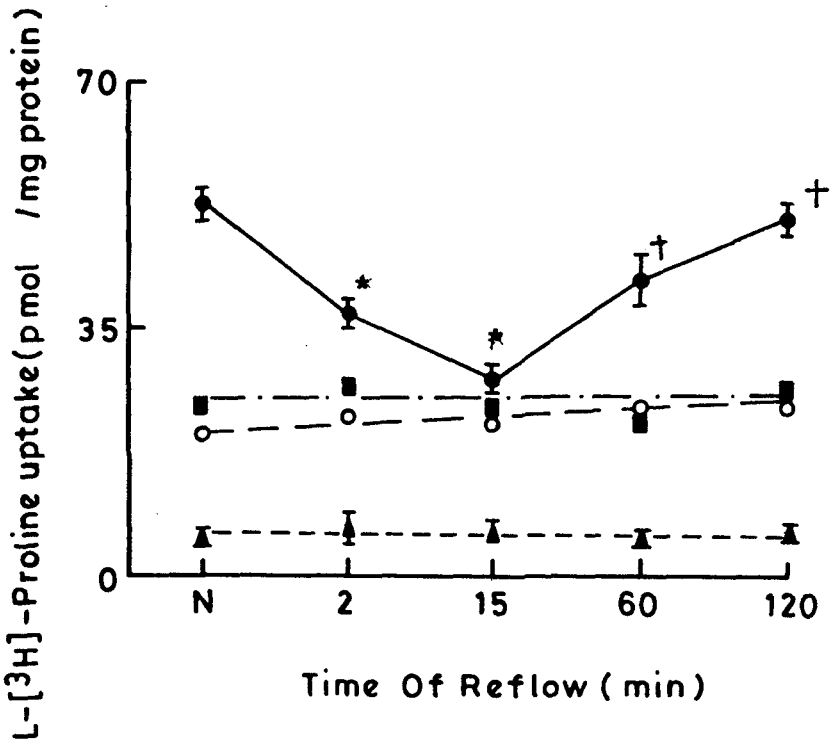


Fig.34: Time course of L-[^{3}H]-pro uptake in presence of Na^+-gradient (—●— , 30s and --o--, 120 min incubation) and in absence of Na^+-gradient (—△—, 30s and -·■·- , 120 min incubation) by BBMV(s) from whole cortex after 30 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

†Significantly different from 15 min reflow $p<0.05$ or higher degree by group t-test.

observed in Na-dependent uptake at the equilibrium after 120 min, both after ischemia and reflow. Similarly Na^+-independent uptakes of both D-glucose and L-proline were not affected by ischemia or reflow (Table 47 & 48).

ii) **Effect of 60 min ischemia and 120 min reflow on the transports of ^{32}Pi and L-[^{3}H]-proline :**

The effect of ischemia and reflow on the transports of ^{32}Pi and L-[^{3}H]-proline was also determined under the conditions known to be morphologically irreversible. The results observed are summarized in Table 49 & 50 (Fig. 35 & 36). The 60 min ischemia showed marked decrease in the initial rate of Na^+-gradient dependent transports of both ^{32}Pi and L-[^{3}H]-proline without affecting the rate of uptake at the equilibrium (120 min). However, 15 min as well as 120 min reflow caused further reductions in the transport rates. There appeared to be no sign of any reflow-induced recovery of transports in 60 min ischemic rats as obtained in 15 or 30 min ischemic rats. These results suggest that 60 min ischemia caused long term irreversible damage to the proximal tubular BBMV(s) (Table 49 & 50, Fig. 35 & 36).

c) Kinetics of ^{32}Pi uptake during ischemia and reflow :

To further characterize the decrease of ^{32}Pi transport

Table 49: EFFECT OF 60 min ISCHEMIA AND REFLOW ON Na+-GRADIENT DEPENDENT ^{32}Pi UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	10 sec	30 sec	120 min
Control	144.14±11.35[a]	377.13±33.23	75.86±9.21
2 min	112.46±1.56[b] (-22%)[c]	154.81±6.86[b] (-60%)[c]	86.65±27.31
15 min	78.43±10.02[b] (-46%)[c]	127.05±8.67[b] (-66%)[c]	90.35±14.63
120 min	66.63±7.74[b] (-54%)[c]	114.44±23.26[b] (-70%)[c]	74.27±17.04

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM of three different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Values in paranthesis are % change from Control.

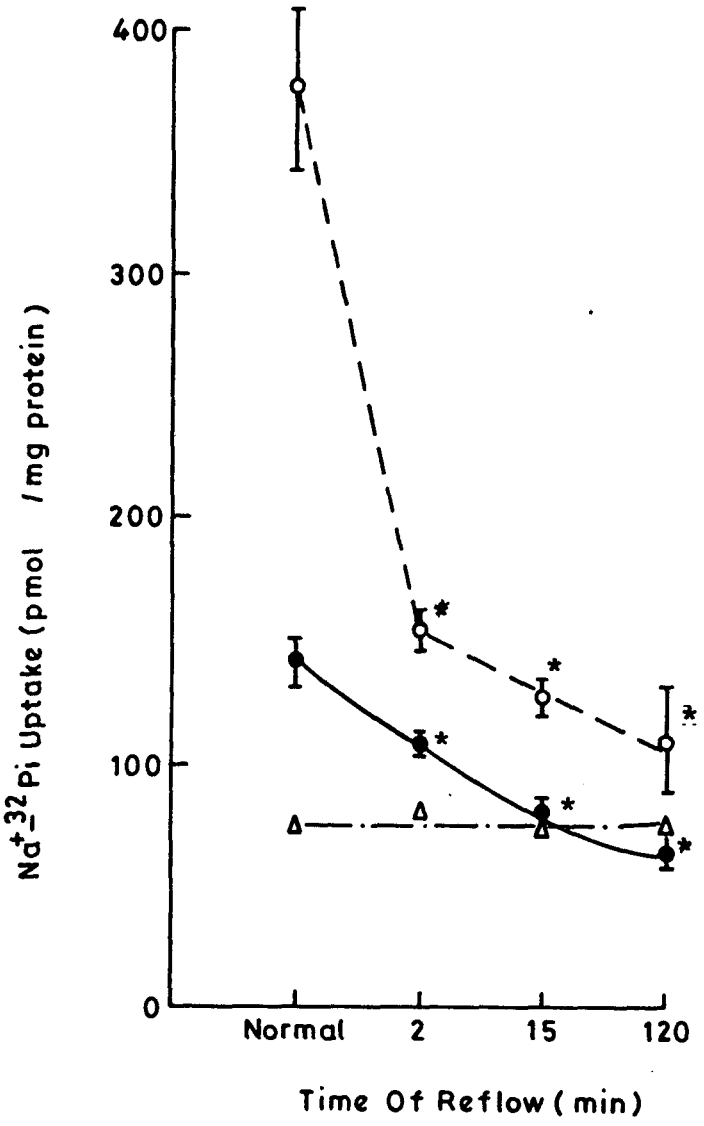


Fig.35: Time course of Na^+-gradient dependent ^{32}Pi uptake by BBMV(s) from whole cortex at 10s (—●—), 30s (--o--) and 120 min (—·△·—) incubation after 60 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

Table 50: EFFECT OF 60 min ISCHEMIA AND REFLOW ON Na+-GRADIENT DEPENDENT L-[^{3}H]-pro UPTAKE BY BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Reflow	30 sec.	120 min
Control	24.35±1.66[a]	6.94±0.095
2 min	11.95±2.01[b] (-51%)[c]	6.57±0.66
15 min	10.47±1.49[b] (-57%)[c]	6.82±0.77
120 min	12.15±4.24[b] (-50%)[c]	5.07±1.24

[a]Uptake (pmol/mg protein) is expressed as Mean±SEM of three different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Values in paranthesis are % change from Control.

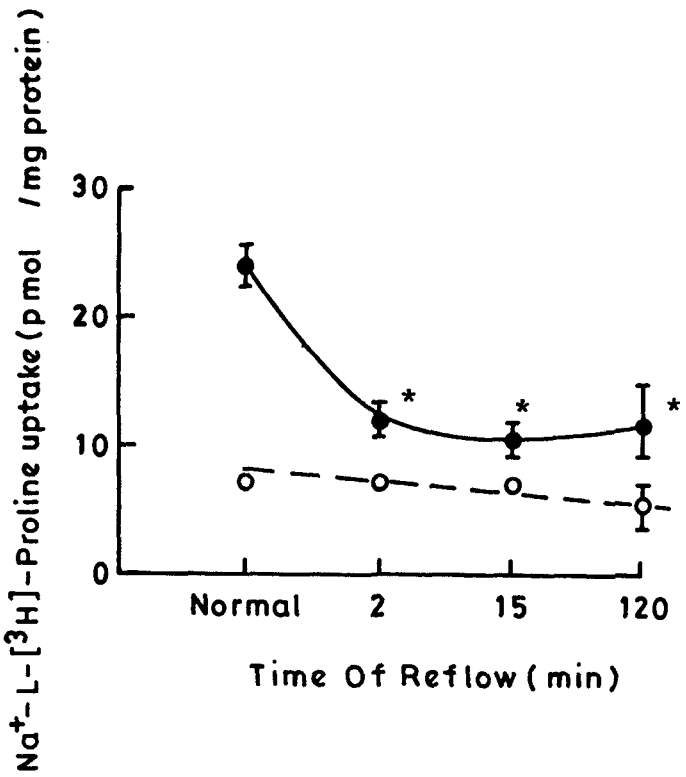


Fig.36: Time course of Na^+-gradient dependent L-[^{3}H]-pro uptake by BBMV(s) from whole cortex at 30s (—●—) and 120 min (--o--) incubation after 60 min ischemia and reflow.

Values are represented as Mean ± SEM for three different preparations.

*Significantly different from Control $p<0.05$ or higher degree by group t-test.

due to ischemia and/or reflow, the uptake was determined at various ^{32}Pi concentrations in the incubation medium. The results indicate that marked reductions due to ischemia (30 min) in Na^+-dependent ^{32}Pi transport were due to decrease in the maximum rate of transport (Vmax), which was more prominent after 15 min of reflow. There was no significant difference in the affinity (Km) of ^{32}Pi transport after 30 min ischemia and also after 15 min reflow (Table 51, Fig. 37). The increase in the transport after 60 min reflow was also due to increase in the Vmax without any significant effect on the Km values. These results clearly indicate that ischemia induced reduction or reflow induced increase in the transport of ^{32}Pi were due to reversible changes occurred in the numbers of active ^{32}Pi-transporters in the BBMV(s).

d) Effect of extra-vesicular Na^+-concentrations in the incubation medium on the transport of ^{32}Pi during 30 min ischemia and upto 120 min blood reflow :

In order to further characterize the effect of ischemia, ^{32}Pi uptake was determined under Na^+-gradient ($Na_o > Na_i$) and non-Na^+-gradient ($Na_o = Na_i$) conditions. As shown in Table 52 the effect of 30 min ischemia and/or reflow on the transport of ^{32}Pi was observed only when inward Na^+-

Table 51: EFFECT OF 30 min ISCHEMIA AND REFLOW ON THE KINETIC PARAMETERS OF Na^+-^{32}Pi UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	Vmax (pmol/mg protein/10 s)	Km ($x10^{-4}$M)
Control	769.23±10.00*	0.085±0.019
2 min	571.90±11.55[a] (-26%)[c]	0.0784±0.034
15 min	333.71±7.87[b] (-57%)[c]	0.074±0.01
60 min	540.93±10.34[a,b] (+62%)[d]	0.09±0.0035
120 min	666.67±8.94[a,b] (+98%)[d]	0.0765±0.001

*Values are Mean±SEM of two different BBMV preparations.

Values are calculated from Lineweaver-Burk Plot (Fig. 37).

[a]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[b]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

[c]% change from Control. [d]% change from 15 min Reflow.

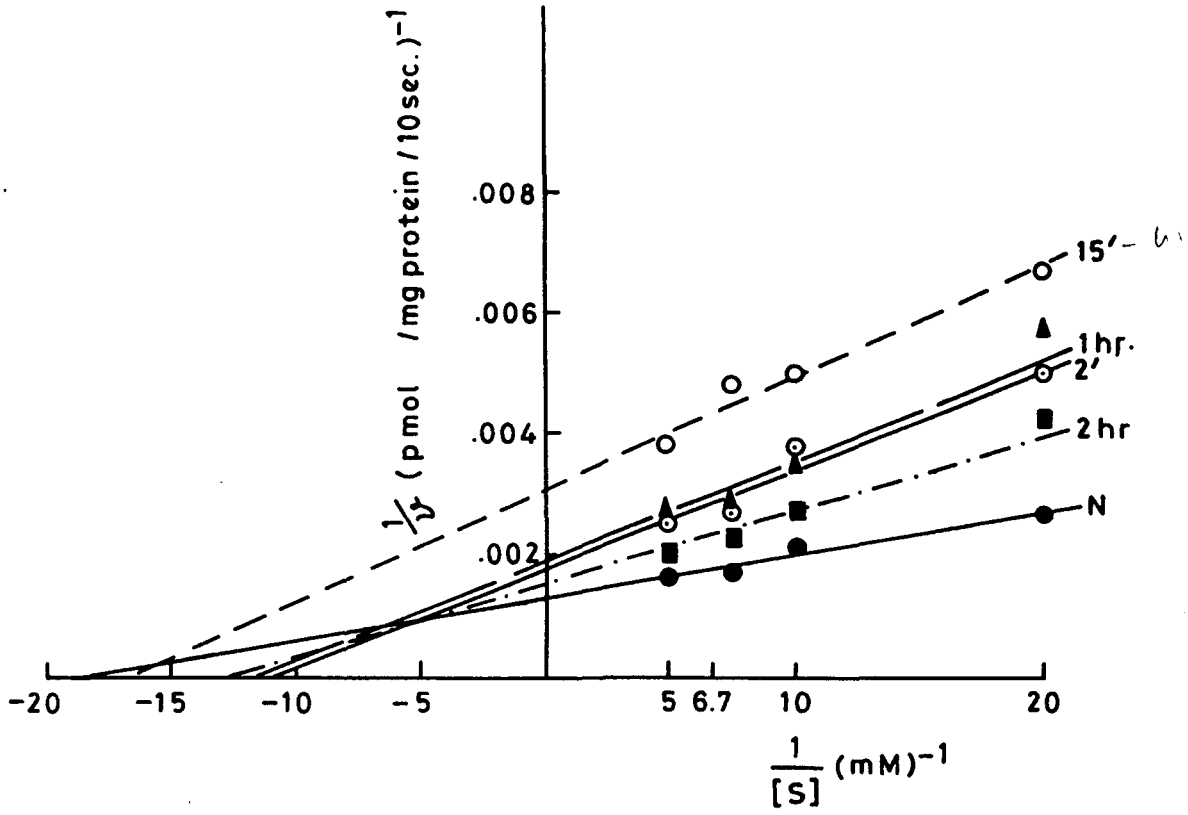


Fig.37: Na^+ gradient dependent ^{32}Pi uptake as a function of phosphate concentration by BBMV(s) from whole cortex after 30 min ischemia and reflow.

Time indicated against each line is time of reflow after 30 min ischemia.

N is sham operated control.

gradient (Na_o > Na_i) was maintained. However, when the gradient was abolished (Na_o = Na_i) the changes in the transport were not observed even though Na^+ was present on both sides of the vesicles. This clearly suggests that only Na^+-gradient transport were affected by ischemia or reflow.

Thus far it has been demonstrated that Na^+-gradient dependent transport in particular of ^{32}Pi significantly decreased by ischemia and was partially restored by the reflow of blood for upto 120 min (Table 46). To further emphasize whether ischemia and reflow alters the properties of ^{32}Pi transport with respect to Na^+-concentration were affected or not the transport of ^{32}Pi was determined at various extravesicular Na^+ concentration (Na_o = 20, 40, 60, and 100 mM in incubation media). The total osmolality and molar concentrations of Cl^- in the media was held constant (300 mOsm, and 100 mM) by appropriate additions of choline chloride as reported by Szczepanska-Konkel et al (192,202). As shown in Table 53, the rate of ^{32}Pi transport increased steadily with increased extravesicular Na^+ concentrations in incubation media in control (sham-operated) rats. However, at all concentration gradients of Na (Na_o > Na_i), the uptake of ^{32}Pi was less in 30 min ischemic rats. Further decrease

in the transport was also observed when blood was allowed to flow for 15 min again at all Na_o (20-100 mM) concentrations indeed proportionally to Na-gradients. The blood reflow of 60 or 120 min showed marked increase in the transport which was also apparently proportional to different extravesicular $[Na_o]$. The graphic analysis of relationship between ^{32}Pi uptake and $[Na_o]$ showed sigmoidal curves during ischemia as well as during the blood reflow of 15 min but not after 60 or 120 min reflow (Fig. 38). It is apparent from the curves that the variations were largely due to alterations in the Vmax. A Hill plot transformation of the data yielded a straight line (Fig. 38, insets A-E) and the calculated $[S]_{0.5}$ were 31.62, 35.48, 25.12, 39.81, and 31.62 for control (A), 30 min ischemic (B) and 15 min (C), 60 min (D), and 120 min (E) RF rats respectively. The values of n were 2.0, 1.67, 1.58, 1.375, and 1.33 respectively for the above curves (Table 54). It appears from this data that about 2 Na^+ were involved in the transport of one ^{32}Pi in control and ischemic rats. However, in the case of 15, 60 and 120 min RF rats less than 2 Na^+ but definetely greater than one Na^+ appeared to be involved for the transport of one Pi molecule. This data suggest that the properties of Pi-carrier with respect to $[Na_o]$ may have somehow altered or

Table 52: EFFECT OF 30 min ISCHEMIA AND REFLOW ON ^{32}Pi UPTAKE ($Na_o > Na_i$ and $Na_o = Na_i$) BY BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Reflow	$Na_o > Na_i$	$Na_o = Na_i$
Control	259.23±12.82[a]	34.29±9.84
2 min	98.12±3.46[b] (-62%)[d]	31.29±1.37
15 min	65.37±8.58[b] (-75%)[d]	33.13±5.62
60 min	159.16±16.81[b,c] (+143%)[e]	33.58±12.25
120 min	207.55±10.43[c] (+217%)[e]	28.87±8.48

[a]Uptake(pmol/mg protein/20s) is expressed as Mean±SEM of two different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

[d]% change from Control. [e]% change from 15 min Reflow.

Table 53:EFFECT OF DIFFERENT EXTRAVESICULAR [Na^+_o] GRADIENT ON 32Pi UPTAKE AFTER 30 min ISCHEMIA FOLLOWEDAND REFLOW BY BBMVs ISOLATED FROM WHOLE CORTEX.

Time of Reflow	20 mM	40 mM	60 mM	100 mM
Control	145.30±9.06[a]	252.75±3.71	426.15±5.23	497.85±1.88
2 min	103.97±3.81[b]	158.19±0.91[b]	247.74±21.58[b]	359.32±15.20[b]
15 min	92.58±0.55[b]	124.33±2.86[b]	179.60±1.40[b]	219.81±17.24[b]
60 min	120.86±8.13[c]	161.39±7.07[c]	223.15±4.82[c]	347.75±11.38[c]
120 min	172.46±2.76[c]	229.25±2.80[c]	267.93±2.44[c]	404.82±0.24[c]

[a]Uptake (pmol/mg protein/20s) is expressed as Mean±SEM for two different BBMV preparations.

[b]Significantly different from Control $p < 0.05$ or greater degree by group t-test.

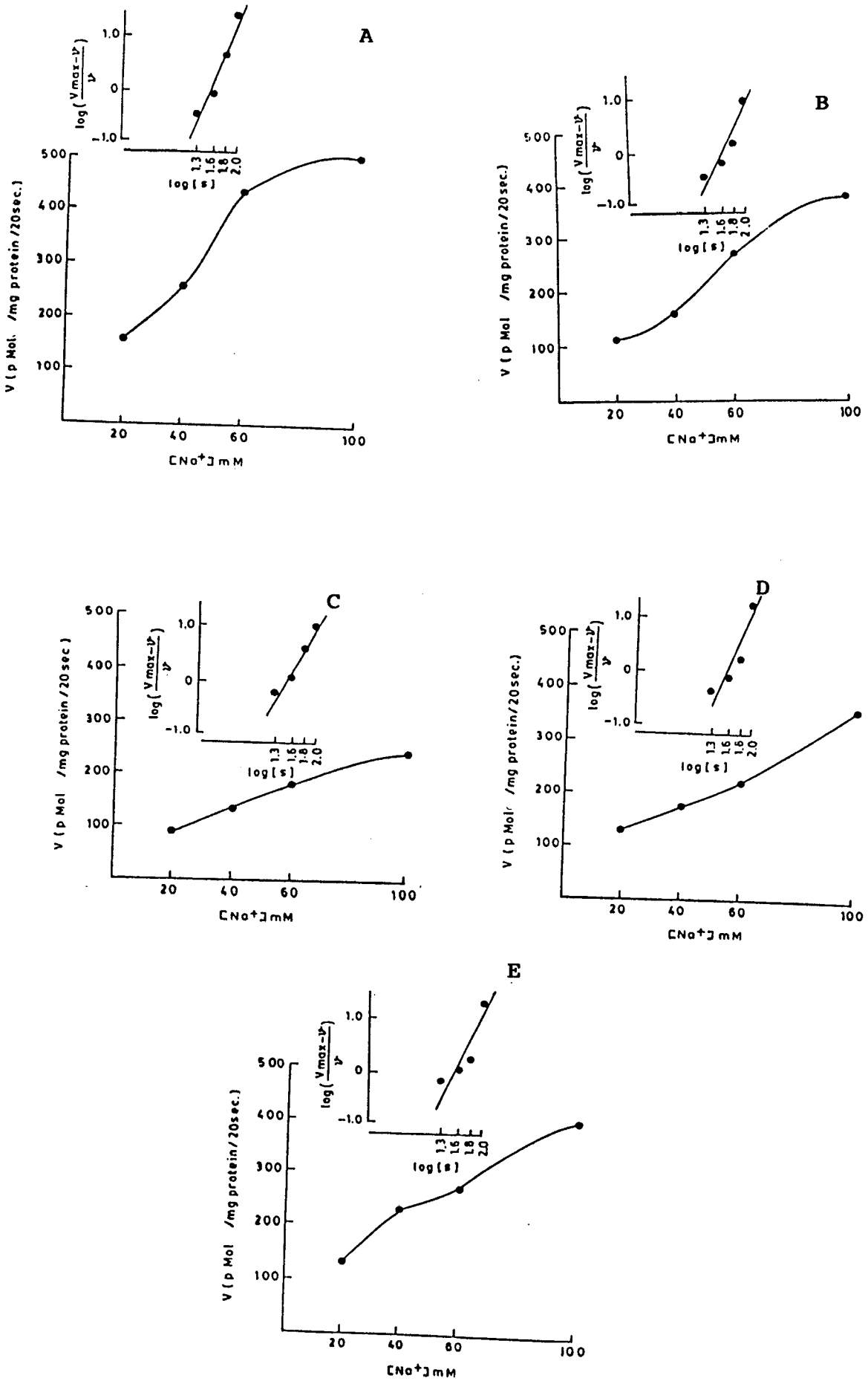
[c]Significantly different from 15 min Reflow $p < 0.05$ or greater degree by group t-test.

Table 54: EFFECT OF 30 min ISCHEMIA AND REFLOW ON KINETIC PARAMETERS OF ^{32}Pi AT VARIOUS $[Na^{+}_{o}]$UPTAKE BY BBMV(s) ISOLATED FROM WHOLE CORTEX.

Time of Reflow	n	$[S]_{0.5}$ mM
Control	2.00	31.62
2 min	1.67	31.62
15 min	1.58	25.12
60 min	1.25	39.81
120 min	1.33	31.62

Values are Mean of two different BBMV preparations.

Values were calculated from Hill's plot (Fig. 38).


A
B
C
D
E
V (p Mol /mg protein / 20 sec.)
[Na+] mM
log [s]
log (Vmax − v / v)

modified during ischemia-reflow induced cellular injury.

e) Effect of T_3 and/or LPD on ^{32}Pi transport after ischemia (30 min) and reflow of 15 and 60 min :

The results of the present studies thus far indicated that ischemia causes specific alterations in the structure and functions of the proximal tubules which were manifested by ischemia-induced (a) decrease in the activity of BBM-marker enzymes, (b) changes in the activities of enzymes belonging to various metabolic pathways and, (c) decrease in the transports of Pi, D-glucose, L-proline across BBMV(s) isolated from the renal proximal tubules. Some of the above ischemia-induced alterations were shown to be partially restored by blood reflow (Table 6 & 8, 18-21, 33-39, 46-48) but only in short duration ischemia (e.g. upto 30 min) and not in long duration ischemia (e.g. 60 min). Thyroid hormone (Thyroxine), generally referred as metabolic hormone showed beneficial effects on the recovery of nephrotoxic ARF (4,24). It has been generalized that these beneficial effects were due to restoration Na^+/K^+-ATPase and cell volume regulation by thyroxine which were affected due to nephrotoxic ARF.

Thyroid hormones (T4/T3) and feeding of a low phosphate

diet (LPD) have been shown to specifically increase BBM's Na-dependent transport of Pi by differential mechanisms (179). Considering the important involvement of Pi in energy-related processes, the following experiment was designed and carried out to determine whether the treatment of T3 and/or the feeding of LPD or their combination can protect ischemia-induced damage or can enhance the recovery of the Pi transport in the proximal tubules. As indicated in Fig. 39 separate groups of animals were placed simultaneously on the different dietary regimens and through out the experiment all groups were studied in parallel (Fig. 39). The animals were first stabilized on NPD for seven days. After day 7, the animals were divided into four groups (12 rats in each group) and the allocation of animals was adjusted so that at this time the groups did not differ in body weight and/or urinary Pi. The rats were then fed with specified diets and treated with T_3 for seven days as indicated. Group I received NPD + 1 ml injection i.p. of vehicle, group II received NPD + 1 ml T_3 i.p. at 100 ug T_3/100 g body weight, group III received LPD + 1 ml injection i.p. of vehicle and group IV received LPD + 1 ml T_3 i.p. at the above dose.

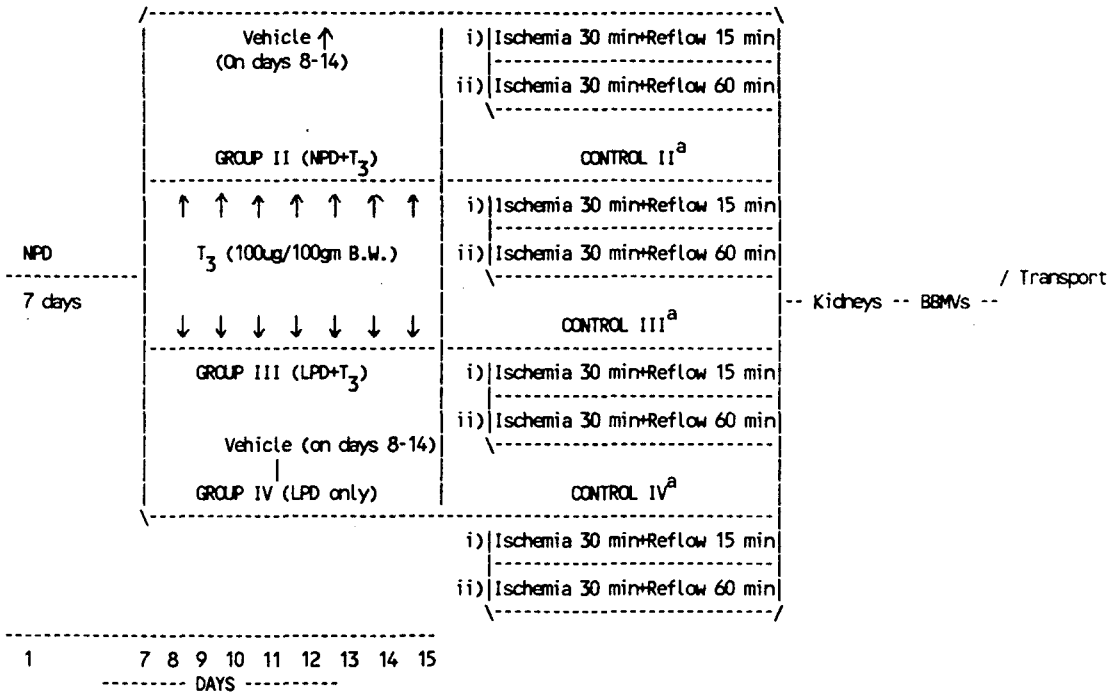


Fig. 39: Flow diagram showing four groups of rats in in vivo experiments: Group I- NPD+1 ml injection of vehicle, Group II- NPD+1 ml injection of T_3, Group III- LPD+1 ml injection of T_3 and Group IV- LPD+1 ml injection of vehicle.

Animals in Groups II and III were given injection of T_3 on indicated days at the given concentration.

[a]Controls I, II, III and IV are the sham operated controls of the respective groups.

The rats (i) with 30 min ischemia + 15 min RF are considered as "ischemic rats", (ii) while with 60 min RF as "recovered rats".

As shown in Table 55 (Fig. 40), 30 min ischemia together with 15 min RF resulted in a significant decrease of Na-dependent Pi transport in NPD-fed rats when the initial uphill uptake was determined at 10s (-40%) or 20s (-47%) as also observed earlier (Table 42). However, blood reflow for 60 min in these rats could restore the uptake of Pi only partially and it was still 75% of control values. In similar experiments, the uptake of Pi was not fully recovered even after 120 min blood reflow (Table 46). In NPD-rats, T_3 treatment for 7 days prior to ischemia showed that the decrease in the Pi transport was significantly much less (-28%) than only NPD-fed rats (-40%). Similarly, prior feeding of LPD-diet to rats for the same period, also showed a decrease in the uptake of Pi to a lesser extent (-27%) compared to ischemic rats fed NPD (-40%) at both initial time periods of 10s and 20s. In the experiment where T_3 treatment was given to rats-fed LPD, an additional increase in the uptake of Pi compared to NPD, NPD + T_3 and LPD rats was observed (Table 54). However, after 30 min ischemia in the rats fed LPD plus treated with T_3, the decrease in Na-dependent uptake of Pi was much less (-23%) (Fig. 40). It should be noted that the rats either treated with T_3 alone or fed with LPD alone and/or given together the initial

Table 55: EFFECT OF 30 min ISCHEMIA AND REFLOW ON Na+-DEPENDENT ^{32}Pi UPTAKE IN BBMV(s) ISOLATED FROM WHOLE CORTEX OF LPD - FED AND T_3 TREATED RATS.

	Uptake at 10s			Uptake at 20s			Uptake at 120 min		
	CONTROL	15 min RF	60 min RF	CONTROL	15 min RF	60 min RF	CONTROL	15 min RF	60 min R
NPD	727.13±59.70[a]	441.29±9.76[b] (-40%)[d]	555.86±22.52[c] (-25%)[d]	1095.17±26.30	575.69±6.95[b] (-47%)[d]	784.76±25.82[c] (-28%)[d]	310.01±12.26	343.19±15.90	308.82±21
NPD+T_3	897.80±31.10	640.15±29.81[b] (-28%)[d]	828.07±21.95[c] (-8%)[d]	1663.18±18.69	937.32±8.27[b] (-43%)[d]	1294.83±17.42[c] (-22%)[d]	269.19±27.17	347.00±26.74	321.80±19
LPD	986.51±20.85	725.30±30.87[b] (-27%)[d]	1032.63±36.95[c] (+5%)[d]	2005.08±39.58	1294.83±17.42[b] (-35%)[d]	1760.73±22.78[c] (-12%)[d]	333.58±14.00	325.85±8.30	327.78±12
LPD+T_3	2363.93±11.59	2006.92±149.14[b] (-15%)[d]	2693.16±63.17[c] (+14%)[d]	3697.34±23.96	2840.32±55.56[b] (-23%)[d]	3840.64±17.20[c] (+4%)[d]	380.07±27.03	387.84±20.59	373.93±15

[a]Uptake is expressed as Mean±SEM of one representative experiment.

[b]Significantly different from respective Contrl $p < 0.05$ by group t test.

[c]Significantly different from respective 15 min RF $p < 0.05$ by grou t-test.

[d]% change from respective Controls.

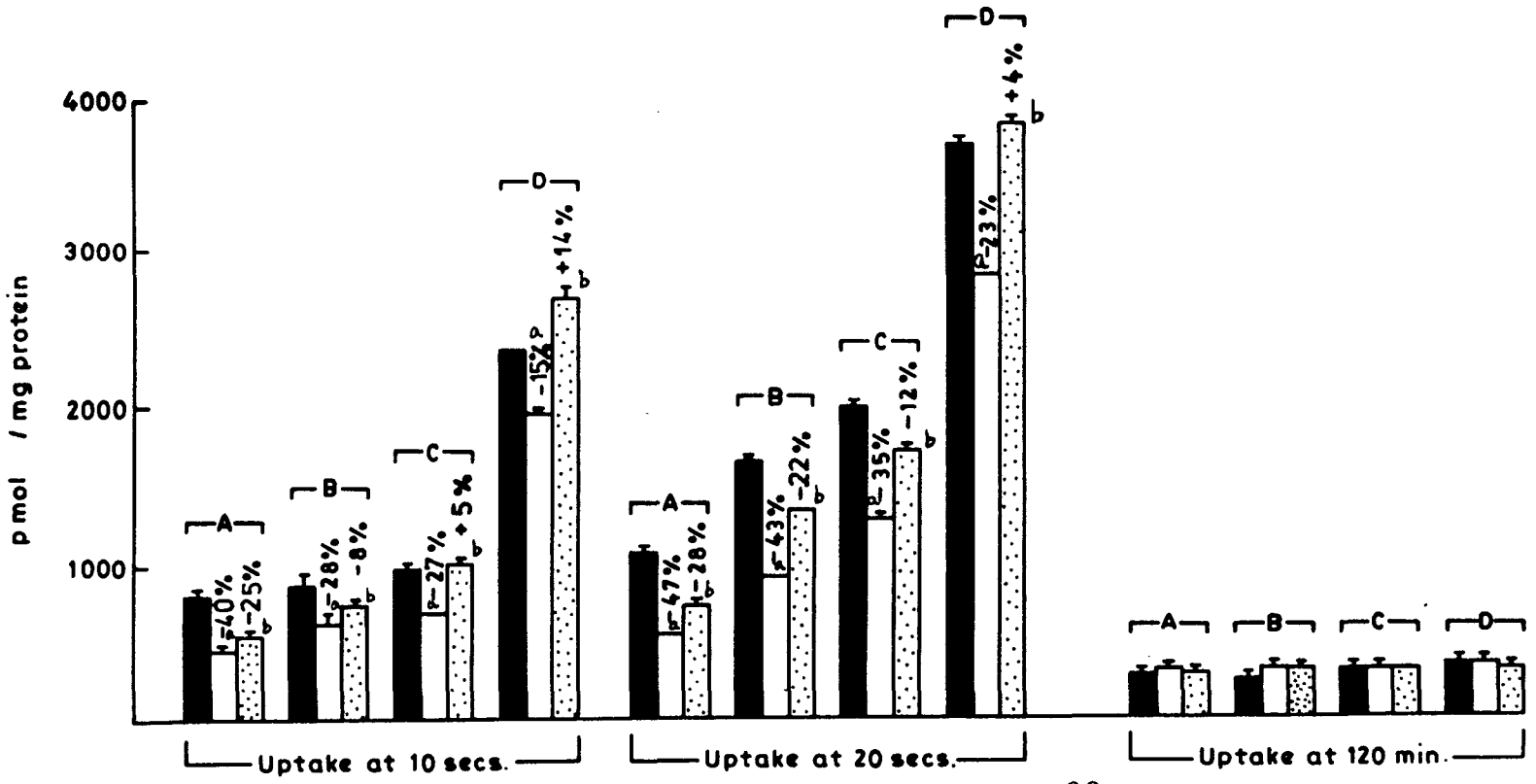


Fig.40: Effect of ischemia (30 min) on the Na^+ gradient ^{32}Pi uptake in rats fed (A) NPD (B) NPD and T3 treated (C) LPD and (D) LPD and T3 treated by BBMV(s) from Control (■) ischemic (30 min ischemia, 15 min reflow, □) and recovered (30 min ischemia, 60 min reflow, ▨).

Values are Mean ± SEM of one representative experiment.

[a]Significantly different from Control $p<0.05$ by group t-test.

[b]Significantly different from 15 min reflow $p<0.05$ by group t-test.

uptake of Pi were significantly higher compared to NPD rats. The uptake was either almost equal to the NPD-rats even after 30 min ischemia + 15 min RF (in the case of T_3 alone or LPD alone) while it was much higher even after ischemia (in the case of T_3 + LPD together). Blood RF for 60 min in the rats except NPD-rats was successfully reversed the damage caused by ischemia on the transport of Pi and the uptake values were restored to corresponding sham-operated control levels. In all of the rats of different groups the uptake of Pi at equilibrium (120 min) was not changed (Table 54, Fig. 40). Kinetic analysis of the effect of ischemia and/or blood RF indicated a Vmax effect to larger extent with only minor variations in the Km values (Table 55, Fig. 41-44).

The results indicate that treatment of T_3 and feeding of LPD alone and in combination may have reduced the severity of ischemic effects on the transport of Pi. The blood reflow in LPD or T_3 rats could have facilitated a quick recovery in the transport rates.

Table 56:EFFECT OF 30 min ISCHEMIA AND REFLOW ON KINETIC PARAMETERS OF Na^+-DEPENDENT ^{32}Pi UPTAKE IN BBMV(s)ISOLATED FROM THE LPD - FED AND T_3 TREATED RATS.

	Vmax (pmol/mg protein/10s)			Km (x 10^{-4} M)		
	CONTROL	15min RF	60min RF	CONTROL	15min RF	60min RF
NPD	666.67	266.67	333.33	0.04	0.024	0.025
NPD+T_3	800.00	363.64	500.00	0.05	0.029	0.033
LPD	1000.00	500.00	727.27	0.044	0.04	0.036
LPD+T_3	1333.33	800.00	2000.00	0.033	0.033	0.044

Values are calculated from Lineweaver Burk plots (Fig. 41-44) of one representative experiment done in triplicate.

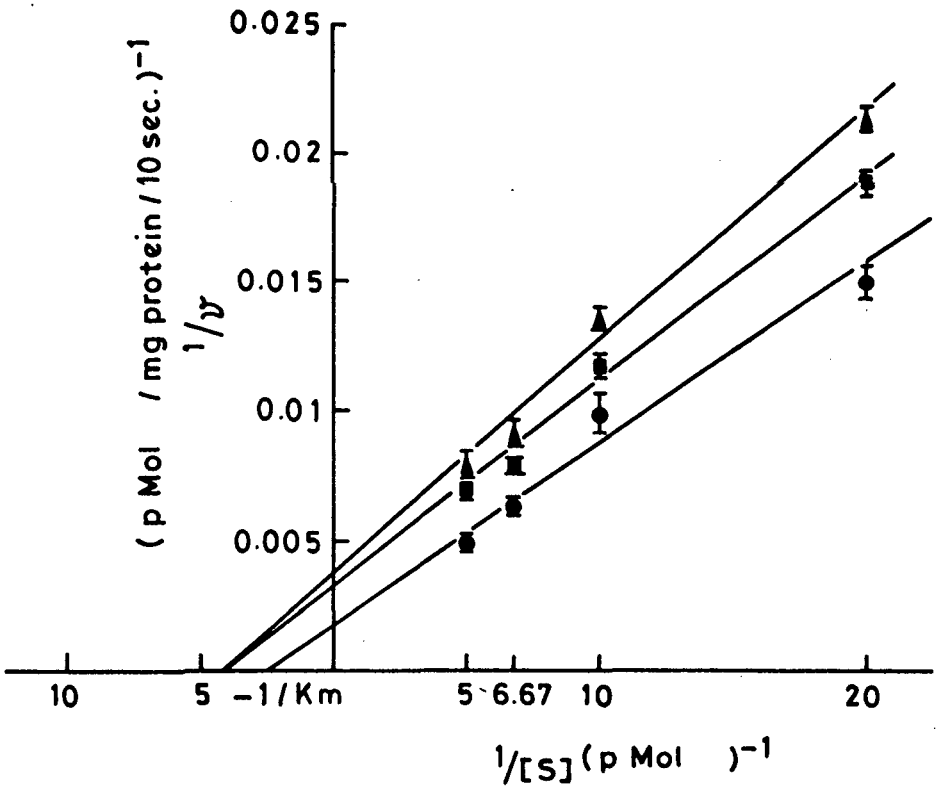


Fig.41: Effect of NPD on Kinetic parameters of Na^+ -gradient dependent ^{32}Pi uptake as a function of phosphate concentration by BBMV(s) from whole cortex of Control (—●—) ischemic (—▲—), and recovered (—■—) rats. (Description of ischemic and recovered rats is given in Fig.40).

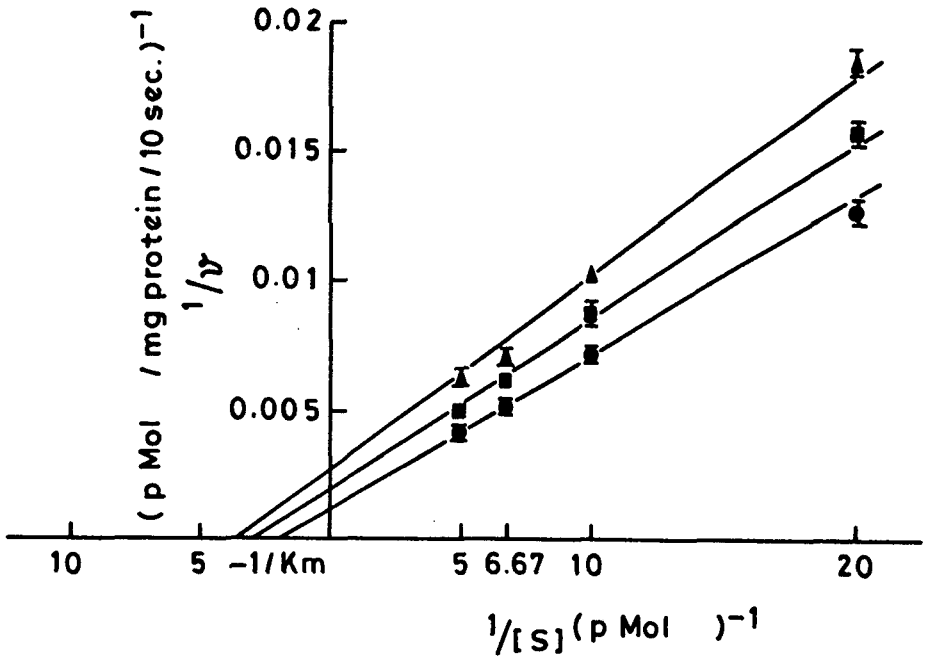


Fig.42: Effect of NPD+T_3 on Kinetic parameters of Na^+ -gradient dependent ^{32}Pi uptake as a function of phosphate concentration by BBMV(s) from whole cortex of Control (—●—), ischemic (—▲—), and recovered (—■—) rats (Description of ischemic and recovered rats is given in Fig.40).

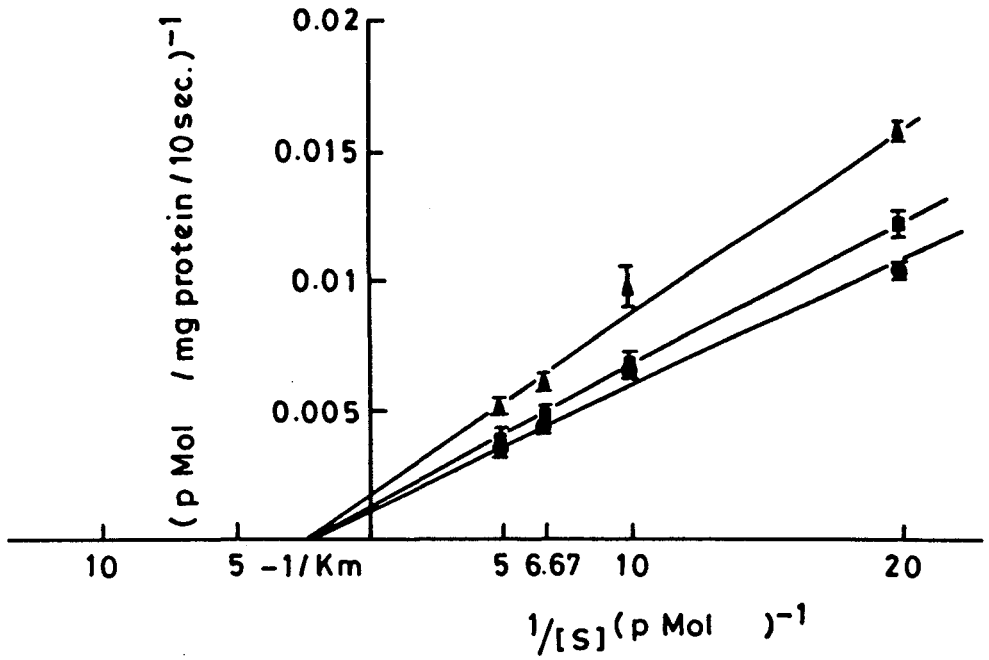


Fig.43: Effect of LPD on Kinetic parameters of Na^{+}-gradient dependent ^{32}Pi uptake as a function of phosphate concentration by BBMV(s) from whole cortex of Control (—●—), ischemic (—▲—), and recovered (—■—) rats (Description of ischemic and recovered rats is given in Fig.40).

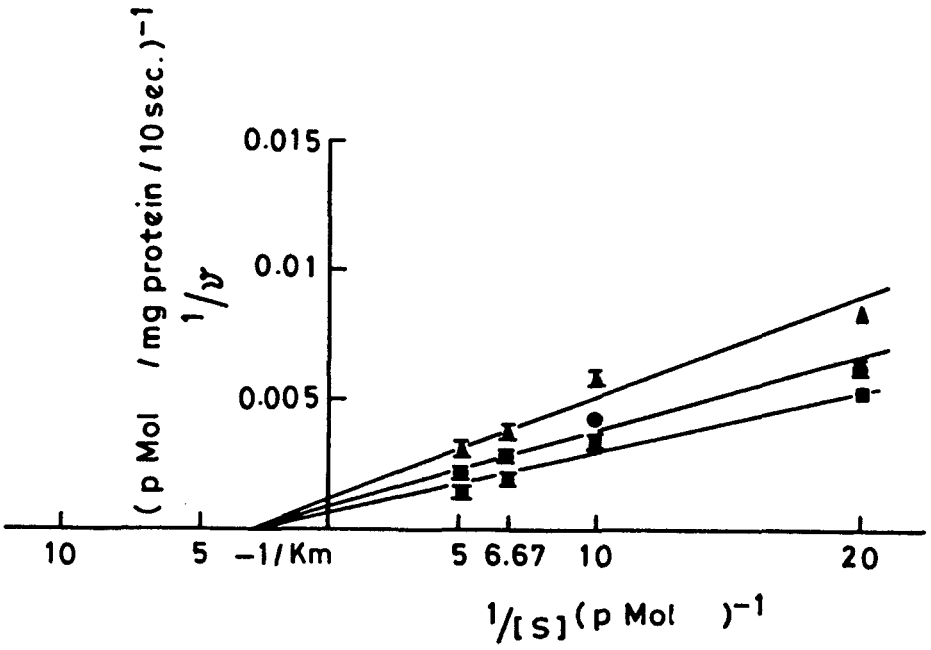


Fig.44: Effect of LPD+T_3 on Kinetic parameters of Na^+-gradient dependent ^{32}Pi uptake as function of phosphate concentration by BBMV(s) from whole ocrtex of Control (—●—), ischemic (—▲—), and recovered (—■—) rats. (Description of ischemic and recovered rats is given in Fig.40).

DISCUSSION - III

Renal ischemia stands as a major cause of acute renal failure (7-9,13,60,62,83,132). Morphologic studies have shown that the proximal tubule particularly its brush border membrane is an earliest and most prominent site of morphologic damage in renal ischemic injury (7-9,132,177). In other cellular and/or biochemical studies ischemia was shown to alter cellular energy metabolism mainly due to lowering of O_2 supply besides other factors which resulted in decreased ATP (60,112). The surface brush border membrane which is damaged due to ischemic or toxic insult resulted in widespread BBM alterations affecting its structural integrity. Surface polarity, membrane fluidity (10,11,133), marker enzymes activities (8,9) are some of the changes induced by ischemia eventually leading to the loss of reabsorptive renal functions. Although renal ischemia is reported to markedly reduce proximal tubule fluid (159), sodium (10), glucose(11) and organic cation (12) reabsorption. The mechanism for such reductions is not yet fully understood. The transport of Pi during renal ischemic injury has not so far been studied, which is especially required for the maintenance of energy yielding reactions as well as in the generation of the currency of energy i.e. ATP. Failure of cells to maintain ATP levels is the hallmark

of ischemia (60,112). Blood reflow to ischemic subjects causes further damage as well as facilitates regeneration processes depending on the duration of blood reflow (7-9,132).

The present studies are designed to carefully examine the effect of ischemia on the transport of Pi together with the transports of L-proline (aminoacid) and/or D-glucose (sugars) under various ischemic and blood reperfusion conditions in the isolated BBMV(s). The effects of ischemia on the transport properties especially of Pi was further characterized to understand the mechanisms of the ischemic effect. Some stimulators of Pi transport such as T_3 and LPD were used to examine whether the effect on Pitransport can be ameliorated.

The results of the present study have indicated that ischemia caused a marked reduction in the transport of ^{32}Pi, L-[^{3}H]-proline and/or D-[^{3}H]-glucose. The reduction in the transport to some extent was dependent on the duration of ischemia. As described in the "Results", Na^{+}-gradient dependent ($Na_o > Na_i$) transport of ^{32}Pi was markedly reduced by 15 min ischemia in the initial phase (5-40s) (Table 42 ;

Fig.29). However, the uptake of 32Pi after equilibration for 120 min was not affected (Table 42). The effect of ischemia on ^{32}Pi transport was affected only when an inward Na^+-gradient ($Na_o > Na_i$) was present while in the absence of Na^+-gradient (when NaCl in the incubation media was replaced by KCl) the uptake was not affected both in the initial phase or at the equilibrium. Na-gradient dependent transport of ^{32}Pi after 30 min ischemia was moderately decreased in comparision to 15 min ischemia. In prolonged 60 min ischemic injury, the ^{32}Pi transport was even higher than in 15 min ischemic conditions. It should be noted that Na-independent uptake of ^{32}Pi both at 30s and at 120 min was increased by 30 min and to a greater extent by 60 min ischemia (Table 43). When net Na-gradient dependent uptake of ^{32}Pi was calculated (Table 44; Fig. 30)the transport of ^{32}Pi was found to be decreased progressively with increased duration of the ischemic injury. This was apparent by % overshoot values which were also decreased progressively after 15, 30 and 60 min ischemia (Table 42). The transport of ^{32}Pi determined in the BBMV(s) isolated from contralateral kidneys was increased markedly more so in 15 min than in 30 or 60 min ischemic rats (Table 42). Thus a compensatory increase might have followed by the remanent

kidney to maintain a positive Pi balance for the cellular need. Kinetic studies revealed that the effect of ischemia on ^{32}Pi uptake was mostly due to the decrease in the number of active transporters. However, alterations in the affinities were also apparent but only to a lesser degree than Vmax changes. Under similar conditions Na-gradient-dependent transport of L-Proline was also decreased by the ischemia however, differentially in comparision to ^{32}Pi transport. While Pi transport was maximally affected by 15 min ischemia, L-proline uptake was decreased significantly both after 15 and 30 min ischemia (Table 45; Fig. 31). Unlike ^{32}Pi transport, L-proline transport at equilibrium, in the absence of Na-gradient and in the contralateral BBMV(s) were not changed by ischemia. The differential effects of ischemia on ^{32}Pi and L-proline transporters appeared to be due to their different locations in the BBM and/or may be due to their differential susceptibilities to the ischemic injury. The decrease in L-proline transport was not in agreement with the earliar observations (11)in which alanine transport was not affected by the ischemia.

Blood reflow to ischemic rats is known to initiate regeneration processes and to restore partially or fully some of the kidney functions (7-9,83,132). However, it seems

to be dependent on both the duration of ischemia as well as on the duration of reflow. The results showed that initial reflow for 15 min further declined the Na-gradient-dependent uptakes of ^{32}Pi, L-proline and D-glucose in the initial phase without changing uptakes of the equilibrium phase of 120 min and Na-independent uptakes were also not altered either by 30 min ischemia and 15 min reflow.

It has been reported that certain components of ischemic cellular injury develop by early blood reflow (7-9,132). Disturbances in cellular calcium hemeostasis (101-105) and enhanced generation of oxygen free radicals (29,30,34,143-149) were reported during ischemia and early reflow causing adverse effects on cellular membranes leading to decreased transport capacities. Also tubular obstruction, which occurs due cast to formation during ischemia is responsible for the sluggish blood reflow initially, causing further cellular injury (13). Membrane degeneration and its interiorization was also reported during ischemia which could be removed by early reflow (7,132) and leads to further loss of transport functions (10-12) as observed in this study after 30 min ischemia and 15 min reflow. Thus some of the above factors might be responsible for

additional loss of uptakes of 32Pi, L-proline or D-glucose by 15 min reflow after 30 min ischemia.

Prolonged reflow of blood for 60 min reversed the decreasing effects of ischemia on the transports of ^{32}Pi, L-proline, and D-glucose.The uptake of ^{32}Pi was 24-32% higher after 60 min reflow compared to 15 min reflow. Further reflow for 120 min profoundly increased Na-gradient dependent transport of ^{32}Pi than 15 min reflow resulting in the restoration of ^{32}Pi transport upto 60-80% of sham-operated control values (Table 46; Fig. 32). Similar results were also obtained for D-glucose (Table 47; Fig. 33) and L-proline (Table 48; Fig. 34) uptakes. Thus, it appears that 30 min ischemia causes reversible damage to membrane transporters which could be partially restored by a 120 min reflow. It is apparent from the kinetic studies that the decrease by 30 min ischemia or recovery by the reflow in ^{32}Pi transport were largely due to alterations in the Vmax, and to a lesser extent in the Km values (Table 51; Fig. 37).

The effects of 60 min ischemia and subsequent blood reflow for 15 or 120 min on the transports of ^{32}Pi (Table 49; Fig 35) and L-proline (Table 50; Fig. 36) were quite different from those of 30 min ischemia and subsequent blood

reflow values. The initial rates of 32Pi and L-proline uptakes declined after 60 min ischemia were further depressed by both 15 and 120 min blood reflow (Fig. 35 & 36). As there was no sign of recovery it appeared that 60 min ischemia produced irreversible damage to the transporters which could not be repaired even to a lesser extent after 120 min reflow.

The actual site and detailed mechanism of ischemic injury or recovery by blood reflow are not clearly known. However, several lines of evidence indicated that ischemia caused alterations in BBM which was documented by morphological studies (7,13,74,130). It has been reported that the BBM is both lost into the lumen and internalized within the cell (7). The extent of membrane loss or duration of time required for regeneration is dependent upon the degree of ischemic injury (8). Biochemical evidence indicated an alteration in the BBM lipid composition(10,133-140). The synthesis of phospholipids were depressed due to ischemia while it was increased in the regeneration phase by the reflow (8,9,134,136). These changes may result in the altertions of membrane fluidity as well as polarity as suggested by Molitoris (136-139). Transports of D-glucose

and 32Pi were shown to be differentially affected by the changes in the membrane fluidity (171,179). Synthesis and turnover of some other cell organelles may also be affected by variation in the lipid composition. Changes in serum as well as BBM phospholipids and cholesterol were observed in the present studies (Table 1-3,41). These can be regarded as biochemical abberation responsible for altered structural and functional integrity of proximal tubule and/or its BBM (136). Inadequate oxygen supply and lack of metabolites during ischemia resulted in the decreased ATP levels required for active transports of Na^+ and other Na-dependent transport e.g. glucose (60,117). It has been demonstrated that 5 min ischemia caused 50% reduction in renal cortical ATP level, while in 20-50 min ischemia it was only 25% depressed (12). Reduced ATP levels with decreased Na^+/K^+ ATPase activity (12) may have caused decrease in ^{32}Pi transport and transport of other solutes.

Alterations in the intrinsic properties of transporter molecules due to ischemia were suggested (10). Reduced phlorizin binding was demonstrated when Na-dependent glucose transport was lowered by ischemia indicating reductions in the number of transporting glucose (11).

The present studies also addressed the question whether ischemia somehow modified the Pi-transporter which may have caused decrease in the uptake. The properties of ^{32}Pi-transporter with respect to $[Na^+_o]$ during ischemia-reflow were studied. It was observed that the effect of ischemia to decrease ^{32}Pi transport was observed only when an inward Na-gradient ($Na^+_o > Na^+_i$) was present (Table 52). However, when Na-gradient was abolished ($Na^+_o = Na^+_i$) ischemia did not change the transport of ^{32}Pi. At different extravesicular $[Na^+_o]$, the transport of ^{32}Pi increased steadily with increasing Na concentrations in the control rats as also observed earlier (192,202). However, the transport did not increase at the same rate in ischemic rats with increasing $[Na^+_o]$ and at all Na-gradients studied ($[Na^+_o]$=20-100 mM). The uptake of ^{32}Pi was always lower in ischemic compared to control values (Table 53). It has been suggested that Na^+ serves both as a driving force, in terms of gradient ($Na^+_o > Na^+_i$), for the translocation across BBM, but also as a factor modulating the interaction of Pi with the Na-Pi co-transporter in the BBM (47,224-226). The allosteric modulatory component of Na^+_o action (47,224) likely accounts for sigmoid dependence of Pi transport on $[Na^+_o]$. This sigmoidal dependence was more apparent in controls, after 30

min ischemia and after 15 min reflow but not in 60 or 120 min reflow rats. A transformed Hill plot of the data yielded a straight line (Fig. 37) and the calculated $[S]^{0.5}$ varied between different groups. It was lowest (25.12) after 30 min ischemia + 15 min reflow compared to controls (31.62), where ^{32}Pi transport was maximally decreased. The calculated n values which indicate the number(s) of Na^+ interacting with one Pi for transporting it were 2, 1.67, 1.58, 1.375 and 1.33 for control (sham-operated), and 2, 15, 60, and 120 min reflow after 30 min ischemia, respectively (Table 54). It is apparent that about two Na were interacting with one Pi in control as well as in 30 min ischemic rats. However, it appears that less than two but greater than one Na were involved during reflow period (15-120 min). The data can guardedly suggest that interactions between Pi-transporter and Na might have modified during ischemia and reflow.

Finally preliminary experiments were performed to demonstrate whether the decrease in the ^{32}Pi transport by ischemia can be prevented or the increase in the transport can be enhanced during reflow by using known stimulators of Pi-transport. Thyroid hormone (T_3 or T_4) and feeding of LPD are reported specifically to increase Na-dependent Pi

transport by Vmax effect, however by different mechanism (179). T_3-treatment or LPD feeding alone resulted in the significant increase of Pi transport in the uphill phase and not at equilibrium as shown in Table 55 (Fig.40) as also reported earlier (168,179).

The results showed that 30 min ischemia and 15 reflow (which causes maximum damage) significantly decreases Pi transport in NPD control rats. The decrease in ^{32}Pi transport due to prior T_3 treatment or LPD feeding was tended to be to be less in T_3 and LPD rats but not significantly than NPD rats. A 60 min reflow in ischemic rats reversed the effect of ischemia on the uptake of ^{32}Pi (Table 55; Fig.40) and apparently there was a greater recovery in ^{32}Pi transport in LPD-rats than NPD-rats (Table 55; Fig.40). However, when the rats were simultaneously treated with T_3 and fed on an LPD, the uptake of ^{32}Pi increased several fold compared to T_3 or LPD alone. Ischemia causes a decrease in the transport but to a lesser extent and the uptake of Pi was still several folds higher than the control or only LPD fed or only T_3 treated rats. Blood reflow for 60 min to T_3 treated + LPD-fed rats showed a speedy recovery and transport of Pi was almost equal or even higher than in respective control rats (Table 55; Fig.40).

The results successfully demonstrate that T_3-LPD combined could reduce in the ischemia induced lowering of ^{32}Pi transport of and blood reflow to these rats was able to cause a speedy recovery. It has been reported that T_3 enhances Pi transport by increasing the activity of Na^+/K^+ ATPase and by synthesizing more transporters while LPD enhances Pi transport by increasing the rate of translocation of transporter due to altered BBM fluidity (179). How T_3 modulated the transport during ischemia and reflow remained to be seen and is a subject of future studies.

SUMMARY

"Acute Renal Failure" is a process in which both kidneys stop their excretory function. The major causes of ARF have been identified as either ischemia or toxic insult to kidney which is manifested by oligaonuria and increased blood urea and creatinine. A variety of experimental models have been used to study ARF, but ischemia was found to be most useful one. Classical concepts regarding the cause of the loss of renal functions after ischemia include : tubular leakage across the damaged epithelium, tubular obstruction by casts formation, and decreased RBF and GFR (13). Mason (1986) proposed a new concept of vascular congestion which causes tubular obstruction as an initiator of ischemic ARF. It is reported to be produced by the aggregation of erythrocytes (13). It has been demonstrated that the tubular congestion due to vascular congestion during ischemic insult was more in the medullary region than the cortical region of the kidney (13,66,68) and was closely related with the degree of the impairment of renal functions (13,61-65). A blood reperfusion after ischemia was able to improve renal function with the removal of mostly medullary congestion (13).

In recent view the mechanism of ARF is thought to be

located at the tubular level rather than at the vascular level(6). The proximal tubule along the length of the nephron is now unequivocally considered to be the principal site of damage caused by ischemic or drug induced ARF. It is believed that obstruction material originates largely from damaged proximal tubular epithelium. During the early phase of ARF exfoliated microvillar brush border membrane (BBM) sloughed into the lumen which might be narrowed down by cell swelling (7). The casts are formed and causes tubular obstruction which lead ultimately to tubular necrosis and kidney functions are altered (1). Both proximal convoluted as well as straight tubules are greately but differentially affected (67). Mason (13) has reported that surface nephrons i.e. superficial cortical proximal convoluted tubules were less affected while deep nephrons ie S_3-subsegments of proximal straight tubules were severely damaged during ischemia. The effect of ischemia was dependent on its duration. A short-term ischemia (5-30 min) causes reversible alterations while prolonged ischemia beyond 60 min causes irreversible damage that can not be repaired even by prolonged blood reperfusion (7-9,60,112).

Renal ischemia results in profound alterations in the

morphology (203), structural integrity (7-9), metabolism and functions (60) of the proximal tubules. The brush border membrane, major functional site in the proximal tubule, is badly damaged both by ischemia and early reflow (7-9).

In ischemia-induced biohemical aberrations, inadequate supply of oxygen and metabolites resulted in severely depleted ATP levels (60,112) and altered Ca^{++}-homeostasis in the renal cortex (104-106). Both aerobic and anaerobic cellular metabolic activities were modulated (60,112,126-128) which were manifested by decreased glycolysis and TCA cycle (126-128). As a result BBM degenerates and BBM-transport functions are impaired (10-12). The activities of some BBM marker enzymes also decreases in the membranes and appeares in the urine (160-161).

In other physiologic alterations of BBM due to ischemia are loss of polarity, redistribution of Na/K ATPase, changes in lipid composition, altered membrane fluidity and loss of selective permeability for certain ions and molecules (134-139). In particular, there was a marked reduction in Na^{+}, and glucose reabsorptions (10-11). The transport of organic cations such as tetraethylammonium was also affected but only moderately (12) while alanine uptake was not altered

(11). The Na-dependent transport of Pi was not characterized in detail during ischemic injury except that of a report by Silverman et. al (227). However, Andreoli et al (142) recently reported that an oxidant injury by H_2O_2 in LLC-PK1, cells lowers Pi uptake in a dose dependent manner. Some of the above effects of ischemia were found to be restored by blood reflow of certain durations (7-9,60). Some limited attempts were made to ameliorate the effect of ischemic or drug induced ARF by certain hormones, drugs or metabolites (14-35,44).

In view of the above, the present studies were carried out to determine in detail the effects of ischemia and reflow on various components of the proximal tubules to gain an insight on the mechanism of ARF and its repair. The following studies were performed and the effects of ischemia and reflow were determined:

(a) On the activities of certain metabolic enzymes especially of carbohydrate metabolism in different kidney tissue zones e.g. cortex and medulla.

(b) On the activities of certain marker enzymes of proximal tubular BBM isolated from renal cortex and from superficial and juxtamedullary cortex.

(c) On the transports of 32Pi, L-(3H)-proline and D-(3H)-glucose in BBMV(s) isolated from renal cortex.

(d) On the transport of ^{32}Pi in BBMV(s) isolated from T_3-treated and/or LPD fed rats.

1. The results indicate that ischemia causes a progressive increase in serum creatinine, phospholipids, cholesterol, and inorganic Pi when produced by occlusion of left renal artery for 15-60 min. The blood reflow for different duration (15-120 min) to ischemic rats showed a reversal of ischemia-induced increase of the serum parameters and they were almost restored back towards normal values after 120 min blood reflow.

2. The activities of marker enzymes of BBM, BLM and other organelles such as lysosomes and mitrochondria were also affected by ischemia however differentially.

(a) The activities of AlkPase and GGTase in the BBMV(s) isolated from whole cortex were decreased in a duration of ischemia dependent manner and the maximum decrease was obtained after 60 min ischemia. The activity of GGTase appeared to be significantly lowered than AlkPase . However, The blood reflow upto 120 min

partially restored the activities of the enzymes (80-90% of control values) in 30 min ischemic rats. However, in 60 min ischemic rats, after 120 min reflow the recovery was much less indicating a greater damage by 60 min than 30 min ischemia (Table 4-9).

(b) Since the activities of enzymes in the homogenates were not different between control and ischemic rats, further analysis indicated that affected enzymes were actually dissociated from the membrane and become the part of the supernatent. The activities were decreased in the particle bound fraction and increased in the supernatent. The decrease in the membrane bound and increase in supernatent enzymes were in proportion to the duration of ischemia(Table 10,11).

(c) Since juxtamedullary cortex is reported to be severely damaged by ischemia than superficial cortex, BBMV(s) were isolated from these tissues from control and ischemic rats. The activities of AlkPase and GGTase were decreased linearly proportional to the time of ischemia in both BBMV-SC and BBMV-JM, however both the enzymes decreased to a greater extent in BBMV-JM than

in BBMV-SC. Reflow studies showed further decline in the activities after 15 min reflow. However prolonged reflow for 120 min resulted in the partial recovery. The reversal of GGTase activity was much slow in BBMV-JM than BBMV-SC. Again, membrane bound enzymes were decreased due to ischemia and appeared in the supernatent, while membrane bound enzymes increased with the reduction in the supernatent due to blood reflow. The appearance and disappearance of the enzymes were in proportion to duration of ischemia or reflow. Kinetic studies revealed that activities were altered largely due to Vmax effect and to a lesser extent due to Km effects(Table 12-23).

3(a) The activities of the enzymes belonging to various metabolic pathways of glucose metabolism e.g. glycolysis, TCA cycle, HMP-shunt pathway and gluconeogenesis were differentially affected by ischemia. The activities of LDH, FBPase, G6Pase and ME were rapidly increased by a brief 5 min ischemia both in the cortex and medulla, however, to different extents while the activities of MDH and G6PDH were decreased under similar conditions. An ischemia of 15 min or longer

duration e.g. 30-60 min caused decrease in the activities of all the metabolic enzymes studied. However, the decrease in the activities varied between cortex and medulla and was in proportion to the duration of ischemia(Table 28-31).

(b) The activities of above metabolic enzymes were also studied in the homogenates prepared from superficial (SC) and juxtamedu-llary cortex (JMC) after ischemia. . The pattern of the effect of ischemia was slightly different from that observed in CH and MH. The effect of different duration of ischemia resulted in the variations of certain enzymes more prominently in either of the cortical tissue. LDH was increased only in JMC after 5 min ischemia while greately decreased in SC-H compared to JMC-H after 15, 30 or 60 min ischemia. MDH was also lowered to a greater extent in SC-H than JMC-H at all ischemic time periods. Both FBPase and G6Pase similarly enhanced in SC-H and JMC-H after 5 min ischemia. The activity of FBPase decreased proportionally by 15, 30 or 60 min to similar extent in both SC-H and JMC-H. However, G6Pase activity was significantly lowered in SC-H compared to JMC-H after

prolonged ischemia (15-60 min). The activities of G6PDH and ME were increased both in SC- and JMC-H after 5 min ischemia, but the increase was greater for G6PDH. The activities of G6PDH were lowered after 15, 30 and 60 min ischemia similarly both in SC and JMC-H. However, ME activity was greately lowered in JMC than SC-H after 15, 30 and 60 min ischemia (Table 28-31).

(c) The effect of reflow on the activities of metabolic enzymes in CH and MH was determined only after 30 min ischemia. The activities of LDH and MDH were greately lowered in MH than CH by 30 min ischemia. Blood reflow causes differential effects on enzyme activities in CH and MH. After 15 min reflow the activity of LDH further declined in CH while a recovery was seen in MH and ischemia induced decrease was reduced. The medullary LDH was fully recovered after 60 min reflow but LDH in CH showed only a small recovery. The activities of MDH both in CH and MH showed the sign of recovery just after 15 min reflow but only partially recovered even after 60 min reflow. The effect of ischemia as well as reflow was greater in the cortex than medulla.

The activities of both FBPase and G6Pase (gluco-

neogenic enzymes) were lowered after 30 min ischemia although to different extents in CH and MH. However, the activities were completely recovered just after 15 min reflow and even increased far greater than control values after 60 min reflow. The activity of G-6PDH was lowered greately in CH while ME activity lowered in MH after 30 min ischemia. After 60 min reflow, the activity of G6PDH was much higher both in CH and MH than control values while the activity of ME recovered to control values after 60 min reflow (Table 34-39).

(d) The activity of BLM marker enzyme, Na/K-ATPase was also decreased but similarly both in CH and MH by ischemia and the decrease was linearly proportional to the duration of ischemia. However, the decrease in the activity was observed to be more in JMC-H than SC-H. A 60 min blood reflow to 30 min ischemic rats resulted in the partial recovery of the enzyme. The activity of acid phosphatase increased with the time of ischemia both in SC-H and JMC-H but to a greater extent in JMC-H than in SC-H (Table 32,33,40).

4. Since renal proximal tubule in general and its brush

border membrane in particular is reported to be damaged by ischemia, the transport properties of various solutes were determined especially ^{32}Pi-transport which has not been determined during ischemia and reflow.

(a) The results indicate that the transports of ^{32}Pi and L-proline were decreased differentially by renal ischemia. Na-gradient-dependent ($Na_o > Na_i$) initial uptake of ^{32}Pi declined sharply after 15 min ischemia, while L-[^{3}H]-proline uptake markedly declined both after 15 as well as 30 min ischemia. Further decrease was not obtained after 60 min ischemia and the effect of ischemia was attenuated. Na-dependent uptakes at equilibrium (120 min) were not altered after 15 or 30 min ischemia but increased after 60 min ischemia. Na-independent ($K_o > K_i$) uptakes of both ^{32}Pi and L-proline (initial and equilibrium) were higher in 30-60 min rats compared to control or 15 min ischemic rats. However, net Na-gradient dependent uptakes of ^{32}Pi (Na-dependent minus Na-independent) were declined proportionally to the duration of ischemia (15-60 min). A compensatory increase in contralateral BBMV(s) from the ischemic rats was observed both at initial uphill

phase and at the equilibrium (Table 42-45).

(b) The effect of blood reflow was studied in 30 min and 60 min ischemic rats showed that Na-dependent transports of ^{32}Pi, L-proline and/or D-glucose were affected differentially. Blood reflow for 15 min further declined the uptakes of the solutes significantly. However, 60 min reflow caused a reversal, and the transports were increased compared to 15 min reflow values. Blood reflow for 120 min resulted in a complete recovery of the transports of L-proline and D-glucose but ^{32}Pi transport was only partially restored. The blood reflow to 60 min ischemic rats, however caused further decrease in the Na-gradient dependent transports of ^{32}Pi and L-proline even after 120 min reflow. No recovery was seen in the uptake values. The results indicate that 60 min ischemia causes long term damages to transporters while 30 min ischemia causes damages which could be partially recovered after blood reflow (Table 46-50).

(c) Kinetic studies revealed that the decrease in ^{32}Pi transport by ischemia and increase due to reflow were largely dependent on Vmax alterations but to a lesser

extent on Km variations (Table 51).

(d) The transport of ^{32}Pi was further characterized during ischemia and reflow. Na-gradient dependent transport ^{32}Pi was altered only when an inward Na-gradient ($Na_o > Na_i$) was maintained. There was no effect of ischemia on ^{32}Pi-transport when Na-gradient was abolished ($Na_o = Na_i$). The Na-dependent transport of ^{32}Pi determined under different extravesicular Na-concentrations($[Na_o]$ = 20-100 mM) showed a sigmoidal relationship between the rate of ^{32}Pi uptake and $[Na_o]$ in control rats. The shape of the curves were modified during ischemia and reflow. The effect was more prominent on Vmax-values. A Hill plot transformation of the data yielded straight lines and calculated $[S]_{0.5}$ were 31.62, 35.48, 25.12, 39.81 and 31.62 for control, 30 min ischemia, after 15 min, 60 min and 120 min blood reflow, respectively. The calculated n values were 2.0, 1.67, 1.58, 1.375 and 1.33 for above respective groups. The data revealed a deviations in the interactions of Na^+ with Pi from 2:1 to less than 2 Na^+ for each Pi during ischemia and especially after 15-120 min reflow where the interaction ratio becomes almost 1:1. Thus it

appears that some intrinsic properties of 32Pi transporters in addition to other factors may have been altered during ischemia and subsequent reflow (Table 52-54).

(e) The effect of T_3 and LPD was determined on ^{32}Pi transport during ischemia and reflow. It appears from the results that both T_3-treatment and to greater extent prior feeding of LPD diet alone checked the severity of ischemic damage to ^{32}Pi transport compared to NPD-control rats. Blood reflow for 60 min resulted in enhanced recovery of ^{32}Pi transport in LPD than NPD rats. However, T_3-treatment together with feeding of LPD resulted in several fold increase in Na-dependent ^{32}Pi transport. The effect of 30 min ischemia on ^{32}Pi was very moderate and rate of ^{32}Pi uptakes were still several folds higher than control NPD rats. The blood reflow for 60 min resulted in quick and complete recovery of ^{32}Pi transport which was not achieved even after 120 min reflow in NPD rats where it was only partially restored (Table 55, 56).

In conclusion, the results of the present study evidently showed that ischemia produced by the occlusion of

renal artery markedly damages the structural integrity and may have altered metabolic and other functions of the proximal tubules. The activities of certain enzymes of carbohydrate metabolism were altered differentially in different kidney tissue zones. It appears that inadequate supply of O_2 and substrates due to termination of blood flow resulted in the alterations of metabolic activity of the cells consequently ATP levels decline in the renal tissues. As a result of ischemic injury (lack of O_2 or ATP) the surface membrane is damaged and may have lost in the lumen. The marker enzyme components either inactivated or dissociated from the membranes and the activities declined. The membrane fluidity may have been affected as a result of changed lipid composition. Finally the transport properties of BBMV(s) alters accordingly. The effects of ischemia was duration dependent and biochemical components of the proximal tubules e.g., BBM-marker enzymes (Table 4-25; Fig. 3-17), metabolic enzymes (Table 26-40; Fig. 18-28) and transport of Pi, proline and/or glucose (Table 42-56; Fig 29-44) were altered to different extent as evident from the results. The blood reflow to certain extent was able to ameliorate the effect of ischemia on different biochemical components. Prior T_3 treatment with LPD-feeding may have beneficial effects in preventing the severity of ischemic damage and in enhancing the restoration of renal transport functions during blood reflow.

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PRESENTATION & PUBLICATION:

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7. **S.J. Khundmiri**, Md. Asghar, F.Khan, S.Salim and A.N.K. Yusufi. Effect of ischemia induced Acute Renal Failure (ARF) on some enzymes of carbohydrate metabolism. (Abstract) Proc. of 64th Ann. Meeting of Soc. Biol. Chem. (India) held at Lucknow (India) from 6-8 Oct, 1995. Abs. No. 440, page 85.